Universität Zürich[UZH]

Epigenetics of the Estrogen Receptors in Women Healthy Aging

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by
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Summary

Life expectancy is currently increasing more rapidly than healthy life expectancy, especially for women. Menopause is a unique biological process in women that is concurrent with aging and further complicates its natural aspects. Steroid hormones contribute to regulate the neuronal activity in the hypothalamus, which is a key player for maintaining vital physiological functions in the entire body. At menopause, steroid hormones sharply decline and remain low afterwards, making women more susceptible to age-related health disorders.

Estradiol (E2), the most potent form of estrogen, exerts several beneficial effects on women's health that are mainly mediated by estrogen receptors (ERs). Although all aging women experience a major decline in E2 levels, not all women experience age-related health disorders to the same extent. It is assumed that the preservation of ERs during aging contributes to maintaining E2 sensitivity and health in advanced age. Among the three mains ERs, named ERα, ERβ, and GPER, the ERα has emerged has the most important ER in maintaining women's health during aging. However, several rapid effects of E2 formerly attributed to ERα could be mediated by the more recently discovered GPER. DNA methylation, the most studied and well-characterized epigenetic mechanism, has been established as a key regulator of ERs expression. Age can induce common methylation changes among individuals as part of a mechanism referred to as "epigenetic clock", which is considered to be the continuation of developmental and reproductive programs beyond the fertile life. Methylation changes at clock cytosine-phosphate-guanine dinucleotides (CpGs) can result in beneficial or detrimental effects on the health status of aging individuals. Studies on mice showed that clock CpGs are mainly localized to key regulatory genetic regions, such as promoters and enhancers. This thesis focussed on the identification in the ER genes of potential methylation mechanisms underlying women healthy aging. Peripheral blood and salivary E2 were collected from 130 healthy middle-aged and older women, recruited in the context of the Women 40+ Healthy Aging Study.

The first aim of this thesis was to establish the protocols combining the Dried Blood Spot (DBS) technology and the bisulfite Next Generation Sequencing (NGS) for assessing ER genes methylation in key regulatory elements. The second aim was to verify whether three regulatory regions of the ERα gene (*ESR1*) were associated with the menopausal status, age, and E2 levels. The third aim was to verify whether levels of *ESR1* and GPER gene (*GPER*) methylation were associated with phenotypes related to dysfunctional estrogen signaling in the suprachiasmatic nucleus (SCN) of the hypothalamus, such as sleep problems and vasomotor symptoms (VMS).

The results of this thesis indicated that DBS and bisulfite NGS were appropriate techniques to evaluate DNA methylation at the regulatory regions of ER genes. Further, lower E2 levels were related to lower methylation of a putative *ESR1* enhancer, particularly at three specific CpGs that were previously suggested to be involved in the negative feedback mechanism underlying the regulation of ERα by E2. Furthermore, increased levels of *ESR1* enhancer methylation were predictive of increased severity of VMS and sleep problems, while there was no evidence regarding the role of *GPER* methylation in VMS and sleep problems. Future research should aim to confirm and advance these findings.

In conclusion, this thesis highlighted potential *ESR1* methylation mechanisms that would underly women healthy aging.

Zusammenfassung

Die Lebenserwartung steigt derzeit schneller als die gesunde Lebenserwartung, insbesondere bei Frauen. Die Wechseljahre sind ein einzigartiger biologischer Prozess bei Frauen, der mit dem Altern einhergeht und dessen natürliche Aspekte noch komplizierter macht. Steroidhormone tragen dazu bei, die neuronale Aktivität im Hypothalamus zu regulieren, der eine Schlüsselrolle bei der Aufrechterhaltung lebenswichtiger physiologischer Funktionen im gesamten Körper spielt. In der Menopause gehen die Steroidhormone stark zurück und bleiben danach niedrig, was Frauen anfälliger für altersbedingte Gesundheitsstörungen macht.
Estradiol (E2), die stärkste Form von Östrogen, hat mehrere positive Auswirkungen auf die Gesundheit von Frauen, die hauptsächlich durch Östrogenrezeptoren (ERs) vermittelt werden. Obwohl der E2-Spiegel bei allen alternden Frauen stark abnimmt, leiden nicht alle Frauen in gleichem Maße an altersbedingten Gesundheitsstörungen. Es wird angenommen, dass die Erhaltung der ERs während des Alterns dazu beiträgt, die E2-Sensibilität und Gesundheit im fortgeschrittenen Alter zu erhalten. Unter den drei Haupt-ERs mit den Namen ERα, ERβ und GPER hat sich ERα als wichtigster ER zur Erhaltung der Gesundheit von Frauen während des Alterns herauskristallisiert. Allerdings könnten mehrere rasche Effekte von E2, die früher ERα zugeschrieben wurden, durch das kürzlich entdeckte GPER vermittelt werden.
Die DNA-Methylierung, der am besten untersuchte und am besten charakterisierte epigenetische Mechanismus, hat sich als ein Schlüsselregulator der Expression von ERs etabliert. Das Alter kann gemeinsame Methylierungsveränderungen bei Individuen als Teil eines als "epigenetische Uhr" bezeichneten Mechanismus‘ hervorrufen, der als Fortsetzung von Entwicklungs- und Reproduktionsprogrammen über das fruchtbare Leben hinaus angesehen wird. Methylierungsveränderungen an Cytosin-Phosphat-Guanin-Dinukleotiden (CpGs) der Uhr können zu vorteilhaften oder nachteiligen Auswirkungen auf den Gesundheitszustand alternder Individuen führen. Studien an Mäusen zeigten, dass CpGs der Uhr hauptsächlich auf wichtigen regulatorischen genetischen Regionen wie Promotoren und Enhancern lokalisiert sind. Diese Arbeit konzentrierte sich auf die Identifizierung potenzieller Methylierungsmechanismen, die dem gesunden Altern von Frauen zugrunde liegen, in den ER-Genen. Peripheres Blut und Speichelfluss E2 wurden von 130 gesunden Frauen mittleren und älteren Alters gesammelt, die im Rahmen der Women 40+ Healthy Aging Study rekrutiert wurden.
Das erste Ziel dieser Arbeit war es, die Protokolle zu erstellen, die die Dried Blood Spot (DBS)-Technologie und das Bisulfit Next Generation Sequencing (NGS) kombinieren, um die Methylierung der ER-Gene in wichtigen regulatorischen Elementen zu bewerten. Das zweite Ziel war die Überprüfung, ob drei regulatorische Regionen des ERα Gens (ESR1) mit dem Menopausenstatus, dem Alter und den E2-Werten assoziiert sind. Das dritte Ziel war zu überprüfen, ob die Methylierung der ESR1- und GPER-Gene (GPER) mit Phänotypen assoziiert sind, die mit dysfunktionalen Östrogensignalen im suprachiasmatischen Nucleus (SCN) des Hypothalamus zusammenhängen, wie Schlafstörungen und vasomotorischen Symptomen (VMS).
Die Ergebnisse dieser Arbeit deuteten darauf hin, dass DBS und Bisulfit-NGS geeignete Techniken waren, um die DNA-Methylierung an den regulatorischen Regionen der ER-Gene zu bewerten. Darüber hinaus hingen niedrigere E2-Konzentrationen mit einer geringeren Methylierung eines mutmaßlichen ESR1-Enhancers zusammen, insbesondere bei drei spezifischen CpGs, von denen zuvor vorgeschlagen wurde, dass sie an dem negativen

Feedback-Mechanismus beteiligt seien, der der Regulierung von ERα durch E2 zu Grunde liegt. Darüber hinaus waren erhöhte Werte der ESR1-Enhancer-Methylierung prädiktiv für einen erhöhten Schweregrad von VMS und Schlafproblemen, während es keine Hinweise auf die Rolle der GPER-Methylierung bei VMS und Schlafproblemen gab. Zukünftige Forschung sollte darauf abzielen, diese Ergebnisse zu bestätigen und voranzutreiben.
Abschließend wies diese Arbeit auf mögliche ESR1-Methylierungsmechanismen hin, die einem gesunden Altern von Frauen zugrunde liegen würden.

Abbreviations

CpGs	Cytosine-phosphate-guanine dinucleotides
DBS	Dried Blood Spot
dMTase	Demethylases
DNMTs	DNA methyltransferases
E1	Estrone
E2	Estradiol
E3	Estriol
ELISA	Enzyme-linked immunosorbent assay
EREs	Estrogen response elements
ESR1	ERα gene
ESR2	ESR2 gene
Estrogen receptors	ERs
GPER	GPER gene
GnRH	Gonadotropin-releasing hormone
HPA axis	Hypothalamic–pituitary–adrenal axis
HPG axis	Hypothalamic-pituitary-gonadal axis
MRS	Menopause Rating Scale (MRS)
MS	Menopausal status
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
POA	Preoptic area
PSQI	Pittsburgh Sleep Quality Index
SCN	Suprachiasmatic nucleus
STRAW	Stages of Reproductive Aging Workshop
SWAN	Study of Women Across the Nation
VMS	Vasomotor symptoms
WHO	World Health Organization

1. General Introduction

1.1 Women healthy aging

The World Health Organization defines Healthy Aging "as the process of developing and maintaining the functional ability that enables wellbeing in older age" (WHO, 2020). Life expectancy has dramatically increased in the last 180 years, and the average lifespan in the world almost doubled during the 20th century. Life expectancy at birth in the EU was about 69 years in 1960 and about 80 years in 2010, which corresponds to a rate of increase in life expectancy of 2.2 years per decade. However, this dramatic increase in life expectancy did not come with a proportionate increase in quality of life for the elderly. Generally, increased life expectancy has increased the risk of disease, disability, and dementia prior to death. In addition, human abilities, such as memory, cognition, and mobility, decline with age, so that the quality of life for individuals older than 90 years is on average very poor even in the absence of degenerative diseases. Increasing life expectancy without increasing health during aging leads to increased morbidity simply because people live long enough to experience age-related disabilities (Brown, 2015). Women generally live longer than men all around the world, on average by six to eight years, and life expectancy for women is more than 80 years in at least 35 countries. As a result, women are also more likely than men to experience age-related disabilities (Ostan et al., 2016; WHO, 2020).

Aging is characterized by a progressive decline in several physiological functions, including circadian rhythms, reproduction, metabolism, hormonal regulation, and cognition. These functions are largely controlled by the hypothalamus, a critical brain region that connects the central nervous system to the periphery through the endocrine system. Functional alterations in hypothalamic neurons naturally occur during aging. These alterations affect the overall health by increasing susceptibility to various health disorders, such as poor sleep, cardiovascular complications, metabolic disease, loss of thermoregulatory control, immune system impairment, cancer, and psychiatric disease (Gouw et al., 2017; Hatcher et al., 2019; Kim and Choe, 2019). As the hypothalamus assures the connection between the brain and the body, hypothalamic dysfunctions induce a systemic impact that promotes systemic aging. Thus, the hypothalamus is hypothesized to be a key element in the process of aging of the entire body (Kim and Choe, 2019).

Menopause is a unique biological process in women that is concurrent with aging and further complicates its natural aspects (Kim et al., 2018). Steroid hormones, such as estrogen in women and androgen in men, contribute to regulate diverse cellular functions in the entire body, including the hypothalamus (Bae et al., 2019; Ruiz-Cortés, 2012). However, at menopause, steroid hormones sharply decline and remain low afterwards, making women more susceptible to age-related health disorders. Indeed, the epidemiology of age-related diseases between genders changes dramatically after menopause. For instance, women's advantage over men in terms of cardiovascular disease gradually disappears with the occurrence of menopause, and women have more disability and comorbidities than men in every age group after age 60 (Brinton et al., 2015; Ostan et al., 2016; Santoro, 2005; WHO, 2007). At the beginning of the past century, both life expectancy and the average onset age of menopause was slightly over 50 years. Today, women can expect to live until 80 years of

age and even longer, although the average age of menopause has remained in the early 50s (Morrison et al., 2006). Therefore, women may spend more than one-third of their life after menopause (Charandabi et al., 2015). In order to extend women healthy aging, it is important to understand the mechanisms underlying it. One of these mechanisms involves the maintenance of sensitivity to steroid hormones in the women's body (Gouwn et al., 2017; Liu and Shi, 2015; Weiss et al., 2004).

1.2 Sex steroid: definition, production and functions

There are three major classes of steroid hormones: testosterone, estrogen, and progesterone. All steroid hormones are synthesized from cholesterol through a common precursor steroid, pregnenolone. Estrogen and progesterone are the primary female sex hormones, and are produced mainly by the ovaries. Progesterone is involved in the female menstrual cycle, embryogenesis, maintenance of pregnancy, and inhibition of sexual behaviour (Ruiz-Cortés, 2012). Estrogen has not only a critical impact on reproductive and sexual functioning through the hypothalamic-pituitary-gonadal (HPG) axis, but also important regulatory functions in the central nervous system, cardiovascular system, skeletal homeostasis, and lipid and carbohydrate metabolism (Vrtačnik et al., 2014).

1.2.1 Estrogen

Estrogen, or oestrogen, exists in three major forms: estrone (E1), estradiol (E2), and estriol (E3). E1 was first isolated in 1929 from the urine of pregnant women. Some years later, E3 (1930), and E2 (1933) were isolated and purified (Santen and Simpson, 2019). Soon after their discovery, natural and synthetic estrogen was introduced for medical use to treat menopausal symptoms (Stefanick, 2005). Estrogens are mainly produced by the ovaries during the fertile life, while before puberty and after menopause they are produced by extragonadal sites, such as the kidneys, adipose tissue, skin, and brain. E1 is most commonly found in increased amounts in postmenopausal women, while E3 plays a role almost exclusively during pregnancy (Cui et al., 2013). E2 is the most potent form of estrogens, approximatively 10 times as potent as E1 and about 80 times as potent as E3 in its estrogenic effect (Ruiz-Cortés, 2012). Therefore, E2 is commonly used as a supplement to reduce symptoms due to estrogen deficiencies (Cohen et al., 2003; Santen et al., 2020). E2 has endocrine, paracrine, autocrine, and even intracrine effects (Vrtačnik et al., 2014). To exert its endocrine effects, E2 reaches the target sites by the bloodstream. In the blood, 98% of E2 circulates bound to sex hormone-binding globulin, and to a lesser extent, to other serum proteins such as albumin. Only a small fraction circulates as free E2, which is the portion available for estrogenic effects. As free E2 in the blood strongly correlates with salivary E2, levels of available for estrogenic effects can be easily measured in saliva with methods such as immunoassays (Dielen et al., 2019; Wu et al., 1976).

1.2.2 Estradiol and women health

E2 paly critical roles in reproductive and non-reproductive functions in women. The roles of E2 include regulation of cardiovascular physiology, bone integrity, muscle mass, subcutaneous visceral fat, hypothalamic functions, such as circadian rhythms (e.g. sleep-wake

cycles) and homeostasis (e.g. the maintenance of core body temperature) (Roepke et al., 2011; Ruiz-Cortés, 2012). Moreover, E2 promotes synaptic plasticity and neuronal repair, which protect against cognitive decline in females during aging (Brinton, 2012; Cui et al., 2013). Therefore, it is not surprising that variations in E2 levels are associated with health disorders. Declining, fluctuating and low E2 levels are associated with adverse health outcomes across the women's lifespan (Biggs and Demuth, 2011; Santoro et al., 2015). Fluctuations across the reproductive lifespan due to the menstrual cycle are associated with a spectrum of premenstrual symptoms including headaches, abdominal bloating, cramping, breast tenderness, weight changes, irritability, decreased concentration, depression, and anxiety (Biggs and Demuth, 2011). During the perimenopausal phase, which encompasses the years preceding menopause, declining and fluctuating levels of E2 are associated with menopausal symptoms, such as hot flashes and night sweat, also called vasomotor symptoms (VMS), vaginal dryness, poor sleep, and depressed mood (Al-Safi and Santoro, 2014; Reed et al., 2007). These and other health disorders such as cardiovascular diseases, osteoporosis, and increased cognitive decline occur during the postmenopausal phase and they have been associated with lower levels of E2 (Catenaccio et al., 2016; Luine, 2014; Lizcano and Guzmán, 2014; Monteleone et al., 2018).

Although all aging women experience major hormonal changes, not all women experience declining physiological functions and the aforementioned health disorders to the same extent (Liu and Shi, 2015; Thurston and Joffe, 2011). The majority of the beneficial effects of E2 are mediated by the estrogen receptors (ERs) (Arnal et al., 2011; Deroo and Korach, 2006; Fuentes and Silveyra, 2019). Therefore, it is assumed that the preservation of ERs during aging contributes to maintaining E2 sensitivity and health in advanced age (Gouw et al., 2017; Cui et al., 2013). Up to today, three main subtypes of ERs, the ERα, ERβ, and GPER, have been described.

1.3 The estrogen receptors

The two main receptors for E2 are the nuclear ERα (NR3A1/ESR1) and ERβ (NR3A2/ESR2) (Kim et al., 2018). The ERα was first identified in 1966 in the rat uterus, while ERβ was identified in the rat prostate in 1996 (Kuiper et al., 1996; Toft and Gorski, 1966). These ERs belong to the nuclear receptor (NR) family of transcription factors, and mediate the majority of biological actions of E2 (Heldring et al., 2007; Luo and Liu, 2020). Like many other members of the NR family, ERs contain conserved structurally and functionally distinct domains (Figure 1). The central and most conserved domain, the DNA-binding domain (DBD), is involved in DNA recognition and binding, whereas ligands (e.g., E2) bind in the COOH-terminal multifunctional ligand-binding domain (LBD) (Heldring et al., 2007). The amino acid sequence of ERα and ERβ displays 59% sequence identity in their respective LBD, which represents a significant difference, resulting in higher transcriptional activity of ERα compared to ERβ (Delaunay et al., 2000, Paterni et al., 2014).

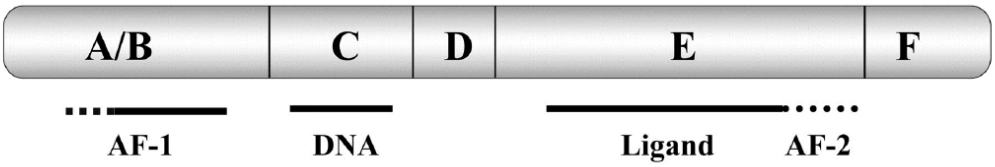

Figure 1: Structure of ERα and ERβ (Nilsson et al., 2001).

In addition to the classical ERα and ERβ, a G-protein-coupled membrane receptor (GPR30), has been described more recently (Figure 2) (Gourdy et al., 2018). The existence of a plasma membrane-associated estrogen binding site was first demonstrated in the 70s in endometrial and liver cells (Pietras et al., 1977). In 1997, GPR30 was identified in the ER-positive breast cancer cell line MCF-7. Because multiple studies showed that this membrane receptor is activated by estrogen, GPR30 was recently renamed G protein-coupled estrogen receptor (GPER) (Zimmerman et al., 2016). Figure 2 shows the structure of classical G-protein coupled receptors. The protein is composed of three main parts (Figure 2): the extracellular region (N-Terminus, ECL2), the transmembrane region (TM region) and the intracellular region (C terminus and ICL2). The extracellular region modulates the access of the ligand, the transmembrane region facilitates ligand binding and transduces the signal by changing the conformation of the intracellular region (Venkatakrishnan et al., 2013).

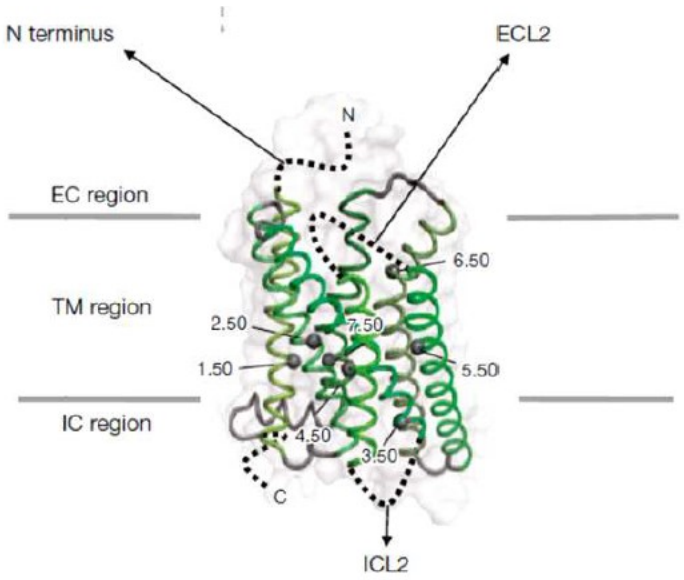

Figure 2: Structure of classical G-protein coupled receptors (Venkatakrishnan et al., 2013).

1.3.1 Genomic signaling pathway

In the classical ER signaling pathway, E2 binds to an inactive ERα or ERβ within the cytoplasm of the cell. The binding of E2 to ERα or ERβ causes conformational changes that enable receptor dimerization, translocation to the nucleus, and binding to the estrogen response elements (EREs) located in or near the promoters of target genes (Bean et al., 2014; Vrtačnik et al., 2014). The binding of the E2-ER complex to the DNA acts as a transcriptional activator of gene expression, by facilitating the recruitment of transcriptional factors on the DNA. The complex E2-ER can also influence the expression of genes indirectly through interaction with

other classes of transcription factors (protein-protein interaction), rather than binding directly to the DNA. This mode of action enables activation or repression of genes that do not harbour EREs in their promoter regions (Menazza and Murphy, 2016; Vrtačnik et al., 2014). Finally, the ERα and ERβ can also be activated through ligand independent-pathways, therefore, in the absence of E2 (Vrtačnik et al., 2014). This activation pathway is mainly triggered by phosphorylation on specific residues (e.g., serine and tyrosine) in the receptors themselves, or through their association with coregulators (Fuentes and Silveyra, 2019). The genomic signaling pathway is illustrated in Figure 3I, 3II, and 3IV.

1.3.2 Non-genomic signaling pathway

E2 can also bind receptors localized at the plasma cell membrane, leading to activation of acute signaling pathways often referred to as "non-genomic" or membrane delimited signaling (Menazza and Murphy, 2016). This pathway induces changes in gene expression that occur in seconds or minutes, which are too rapid to be induced by the classical nuclear signaling pathway. Commonly, the activation of membrane ER is associated with the activation of various protein kinase cascades that can eventually lead to indirect changes in gene expression due to the phosphorylation of transcription factors. Several hypothalamic functions, such as stress responses, control of core body temperature, and energy balance, require rapid signaling by E2 (Qiu et al., 2003). Non-genomic estrogen signaling can be mediated by GPER, and certain variants of ERα and ERβ that belong to a subset of membrane-bound ERs (Vrtačnik et al., 2014). The genomic signaling pathway is illustrated in Figure 3III.

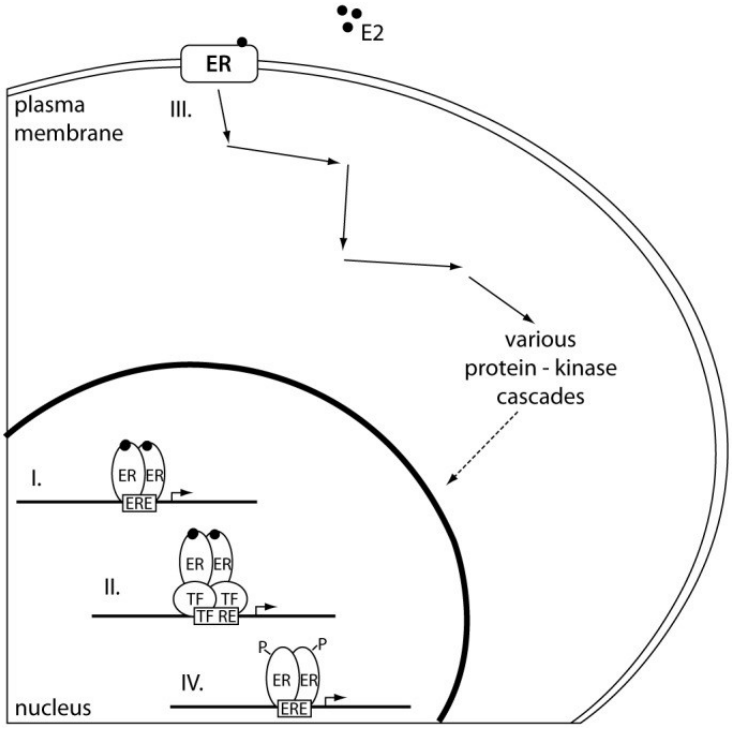

Figure 3: The representation of different mechanisms of estrogen signaling (Vrtačnik et al., 2014). (I.) Direct genomic signaling pathway, considered the classical mechanism of estrogen signaling, promotes target gene expression by binding the E2-ER complex directly to the ERE. (II.) Indirect genomic signaling pathway, E2-activated ERs bind DNA through protein-protein interactions with other classes of transcription factors at their respective response elements. (III.) Non-genomic signaling pathway starts with the binding of E2 to the ERs located at the plasma membrane resulting in the activation of various protein-kinase cascades. (IV.) Ligand-independent signaling pathway causes ER activation and target gene transcription through phosphorylation of ERs or their associated coregulators.

1.3.3 Distribution in the body of ERs

The three ERs are almost ubiquitously expressed and coexpressed in the human body, including the brain. However, there are some differences in their expression patterns. In the brain, the hypothalamus and amygdala emerge as ERα-dominant regions, while some studies describe ERβ as being the dominant form in the hippocampus, although both ERα and ERβ are abundantly expressed in this region (Cui et al., 2013; Gilles and McArthur, 2010). In addition, sex differences are present in the expression levels of ERα and ERβ in hypothalamic nuclei (Gilles and McArthur, 2010). In particular, the suprachiasmatic nucleus (SCN) of women has higher levels of ERα as compared to men (Hatcher et al., 2019). This Sexual dimorphism has been suggested to contribute to the well-known epidemiological data that show longer average life spans for women than men (Gouw et al., 2017). In contrast, there is a lack of overall sex differences between ERα and ERβ expression levels in the hippocampal regions (Gilles and McArthur, 2010). In the periphery, the ERα is primarily expressed in the uterus, epididymis, bone, breast, liver, kidney, white adipose tissue, stroma of prostate, theca and interstitial cells of the ovary, and Leydig cells of testes, while the ERβ is mainly expressed in the colon, testis, bone marrow, vascular endothelium, lung, bladder, epithelium of prostate, and granulosa cells of the ovary. GPER has been observed in the brain, placenta, lungs, liver, prostate, ovary, pancreatic islets, adipose tissue, vasculature, muscle, skeleton, as well as immune cells. The expression pattern of GPER in the human brain is presently unclear (Luo and Liu, 2020).

1.4 Estrogen receptors and women healthy aging

The contribution of ERs to health outcomes is mainly linked to their loss or overexpression (Burns and Korach, 2012). In the context of healthy aging, research on humans and rodents support the role of diminished levels of the three ERs in the exacerbation of age-related health disorders (Cui et al., 2013; Deroo and Korach, 2006; Hatcher et al., 2019; Kelly and Rønnekleiv, 2015; Mott and Pak, 2013; Qiu et al., 2003).

1.4.1 Estrogen receptor alpha

ERα is considered a key player of women healthy aging (Bean et al., 2014; Gouw et al., 2017; Hatcher et al., 2019). In the review by Arnal et al. (2012) on ERs and menopause, ERα is described as “absolutely necessary for most of the beneficial actions of E2”. Indeed, ERα is thought to be the protective ER in several systems during aging (Arnal et al., 2012; Koenig et al., 2017). However, ERα tends to decrease with age, resulting in a decreased ERα/ERβ ratio in several tissues (Kim et al., 2018; Koenig et al., 2017). This may be problematic as the ERα has a higher affinity for E2 than ERβ, and is therefore thought to be fundamental for mediating E2 effects in phases of low E2 levels, such as postmenopause (Bean et al., 2014; Foster, 2012). ERα plays a unique role with respect to the different function of the hypothalamic regions. Within SCN, genomic ERα signaling is responsible for the upregulation of clock genes, such as the period circadian clock 2 (PER2), involved in the maintenance of normal circadian rhythms (Gouw et al., 2017). As they regulate almost every aspect of human physiology, circadian rhythms are critical for survival and overall health (Hagenauer and Lee, 2011; Hatcher et al., 2018). Dysregulation of circadian rhythms during aging is indeed associated with several

health disorders, such as sleep problems, cardiovascular complications, metabolic disease, immune system impairment, cancer, and psychiatric disease (Gouw et al., 2017; Hatcher et al., 2019). Within the arcuate nucleus and the preoptic area (POA), ERα influence reproductive functions by controlling the production of the gonadotropin-releasing hormone (GnRH) (Gouw et al., 2017). Research indicates that the deregulation of GnRH secretion following loss of E2 after menopause may be responsible for the exacerbation of VMS in women (Albertson and Skinner, 2009; Ruiz-Cortés, 2012). In addition, the GnRH significantly reverses aging-impaired neurogenesis in the hypothalamus, hippocampus, and other brain regions (Tang and Cai, 2013). Furthermore, within the supraoptic nucleus and the paraventricular nucleus, ERα signaling also protects neurons from osmotic stress, which in turn strengthens the hypothalamic hypothalamic–pituitary–adrenal (HPA) axis in handling stress. In the limbic system, ERα expression contributes to learning and memory through neurogenesis, regulation of anxiety, neuroprotective effects against glutamate, FeSO4, amyloid-β peptide toxicity, and glucose deprivation (Gouw et al., 2017).

Both the ERα and ERβ signaling in the hippocampus contribute to preventing Alzheimer's disease (Paterni et al., 2014). However, animal studies showed that in advanced age, decreased ERα/ERβ ratio, due to a loss of ERα, is associated with cognitive impairments, such as poor memory (Bean et al., 2014). ERα appears to be the dominant receptor preventing Parkinson's disease, because of its major protective role of dopaminergic neurons in the striatum and the substantia nigra (Cui et al., 2013; Paterni et al., 2014). Clinical studies on osteoporosis indicate that expression of ERα, but not ERβ, is essential in promoting bone-protective actions and bone formation (Deroo and Korach, 2006; Hertrampf et al., 2008). A greater role of ERα compared to ERβ in protecting cardiovascular functions has also been reported (Deroo and Korach, 2006). Finally, animal and human studies showed that decreased ERα/Erβ ratio, due to the loss of Erα during aging, makes aged female mice and postmenopausal women more susceptible to inflammatory diseases, and, in older female mice, it reverses the antioxidant effect of E2 to a pro-oxidant profile (Koenig et al., 2017; Novella et al., 2012).

1.4.2 Estrogen receptor beta

The contribution of ERβ to women health during aging may be less pronounced than the contribution of ERα. However, ERβ has a key role in preventing several cancers. In many breast cancers, ERα activation by estrogens is generally considered responsible for enhancing proliferation, whereas ERβ exerts an antiproliferative effect. In human malignant pleural mesothelioma, ERβ was found to modulate cell proliferation by effectively inactivating the epidermal growth factor receptor, thus exerting a tumor repressive effect, and a similar function was also observed in renal cell carcinoma cell lines. Loss of ERβ expression is generally found in colorectal cancer, and the degree to which it is reduced correlates with an increasingly poor prognosis. ERβ was demonstrated to have a protective effect against the development and progression of colon cancer by inhibiting cell proliferation in vitro and tumor formation in vivo (Paterni et al., 2014).

Besides the role of ERβ in cancers, this ER may play an important role in preventing obesity, by regulating metabolic pathways and adipose tissue function (Paterni et al., 2014). In addition, E2 can prevent cardiac fibrosis by blocking the fibroblast to myofibroblast transition

via interactions with ERβ (Cui et al., 2013). Finally, as ERβ levels tend to be more stable than ERα in the aging brain, ERβ may have important implications as a therapeutic target for improving cognitive functions (Kim et al., 2018).

1.4.3 GPER

GPER signaling has been suggested to be implicated in almost every system of the human body, and there is accumulating evidence for a protective role of GPER against several age-related disorders, such as hypertension, atherosclerosis, ischemia, reperfusion injury, heart failure, metabolic disorders, cancer, and menopausal symptoms (Cui et al. 2013; Prossnitz and Barton, 2011; Tian et al., 2019; Zimmerman et al., 2016). In addition, several non-genomic effects of E2 formerly attributed to ERα have now been described as GPER-mediated (Prossnitz and Barton, 2011). However, findings on the role of GPER in health and disease are controversial, and there is still a lack of evidence that this ER plays a significant role in mediating endogenous estrogen effects in vivo (Luo and Liu, 2020; Prossnitz and Barton, 2011).

1.5 Epigenetic regulation of estrogen receptor

Levels of ERs are regulated by numerous factors, such as mitogen-activated protein kinase, hormonal/nuclear hormonal pathway, and growth factors pathway. However, epigenetic regulation is the most important mechanism by which the expression of ERs is regulated (Pinzone et al., 2004). In addition, epigenetic mechanisms may also partially mediate the effects of the other aforementioned regulatory factors of ERs (Ianov et al., 2017; Lu et al., 2007; Schwarz et al., 2010; Tsuboi et al., 2017).

1.5.1 Epigenetics and human health

Until the 1950s, the term epigenetics was used to denote the poorly understood developmental events by which the single fertilized egg mature into a complex organism of varied phenotypes. During the past 50 years, the understanding of epigenetic mechanisms as the molecular modifications underlying the regulation of gene expression in eukaryotes has dramatically increased (Felsenfeld, 2014). Today, epigenetics refers to “the study of molecular processes that influence the flow of information between a constant DNA sequence and variable gene expression patterns. This includes investigation of nuclear organization, DNA methylation, histone modification, and RNA transcription. Epigenetic processes can result in intergenerational (heritable) effects as well as clonal propagation of cell identity without any mutational change in DNA sequence” (Nature Research, 2020).
In the last decades, studies have increasingly demonstrated the importance of epigenetics in human health. Epigenetic alterations may result in various disorders, from birth defects and childhood diseases to cancers and age-related disorders (Moosavi and Ardkani, 2016). Contrary to the DNA sequence, epigenetic patterns are cell and tissue-specific, as well as potentially reversible. Therefore, epigenetic marks are also receiving increasing interest as potential biomarkers for prediction, diagnosis and prognosis of various diseases, as well as for the development of new therapies, and the monitoring of their effectiveness (Kronfol et al., 2017; Rasool et al., 2015). The three main epigenetic mechanisms are illustrated in Figure 4.

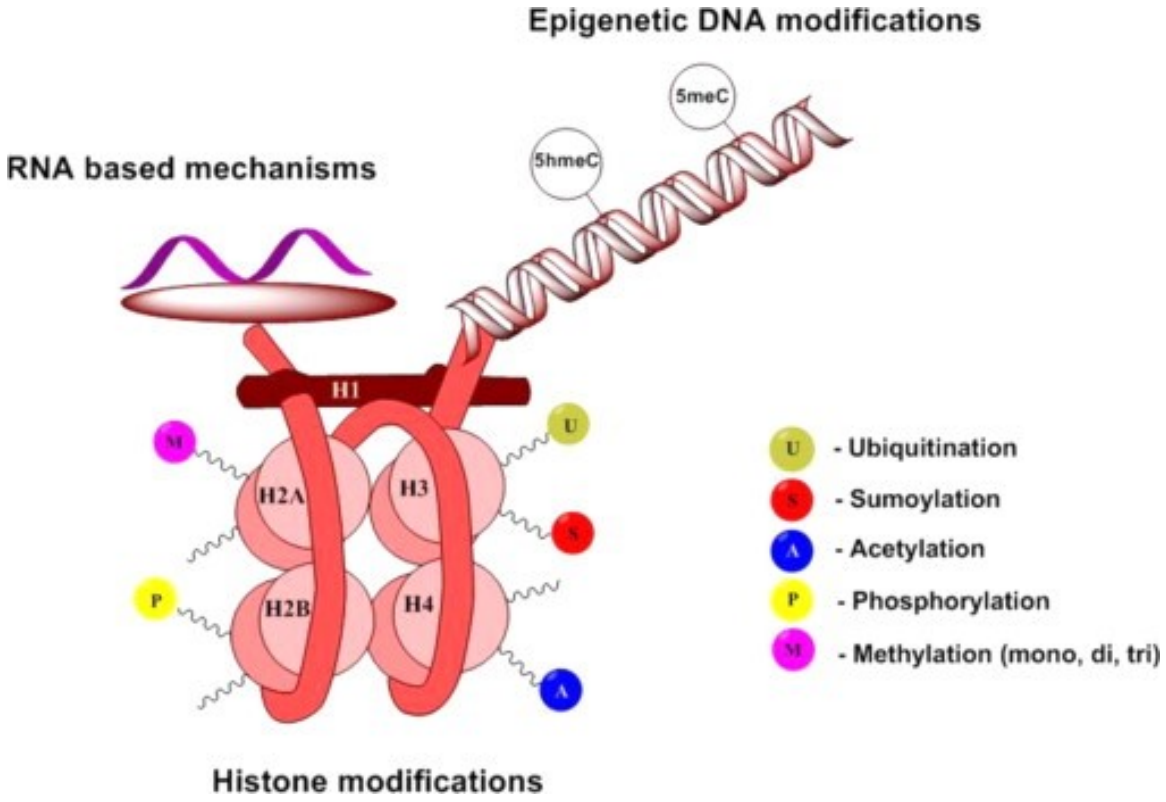

Figure 4: Schematic representation of the main epigenetic mechanisms; DNA modifications, histone modifications, and RNA based mechanisms (Mikhed et al., 2015).

1.5.2 DNA methylation

DNA methylation is the most studied and well-characterized epigenetic mechanism, as aberrant methylation patterns are described in several physical and psychiatric disorders (Tost, 2010). DNA methylation regulates gene expression mainly through the addition of methyl groups to the C5 carbon of cytosines in the cytosine-phosphate-guanine dinucleotides (CpGs) (Moore and Fan, 2013). The addition of methyl groups causes physical binding impedance of transcription factors to the DNA (e.g. RNA polymerase) and repression of transcription (Kumar et al., 2018; Portela & Esteller, 2010). Methyl groups are transferred to the DNA by enzymes called DNA methyltransferases (DNMTs). In mammals, DNA methylation involves DNMT1 and DNMT3s. DNMT1 mainly allows the maintenance of DNA methylation during cell division, while DNMT3s are involved in establishing de novo cytosine methylation. Another important group of methylation-related enzymes referred as to demethylases (dMTase). These enzymes remove methyl residues from the DNA, disrupting methylation patterns previously established (Figure 5) (Gujar et al., 2019).

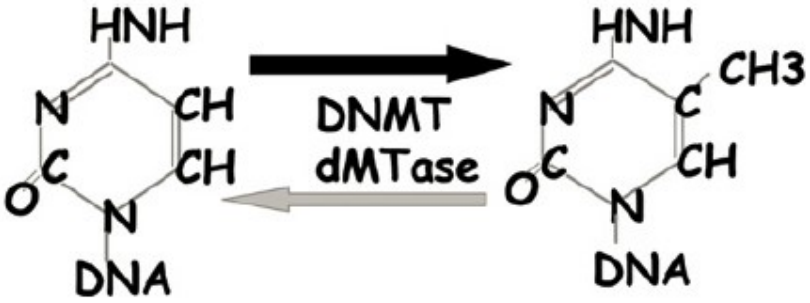

Figure 5: The reversible DNA methylation reaction (McGowan and Szyf, 2010). DNMTs catalyze the transfer of methyl groups from the methyl donor to the DNA. dMTase release the methyl group from methylated DNA.

CpG dinucleotides are concentrated in specific regions of the gene, often referred to as CpG islands. CpG islands are stretches of DNA of about 200-1000 base pairs (bp) long, with a G+C content greater than 50% (Takai and Jones, 2002). CpG islands comprise about 1% of the genome and are generally non-methylated. Methylation of CpG islands is strongly associated with silencing of gene expression (Mohn et al, 2008). Sequences immediately flanking and up to two kilobases away from the CpG islands are known as shores (Martin and Fry, 2018). These regions are less concentrated in CpGs than the islands. However, studies profiling CpG methylation patterns have identified differentially methylated regions (DMRs) in the shores (Moore and Fan, 2013). Like CpG islands, increased methylation at shores is strongly related to gene expression (Irizarry et al, 2009).

Promoters and enhancers are genetic elements that promote gene expression (Andersson and Sandelin, 2019). A promoter is a sequence initiating the process of transcription. The majority of promoters, roughly 70%, reside within CpG islands (Saxonov et al., 2006). Enhancers increase transcription of target promoters, generally by forming a chromatin loop on the promoter that enhances the local concentration of transcription factors (Figure 6) (Pennacchio et al., 2013). Enhancers have intermediate levels of methylation, which indicate dynamic methylation modifications in response to environmental changes (Avrahami et al., 2015, Ordoñez et al., 2019).

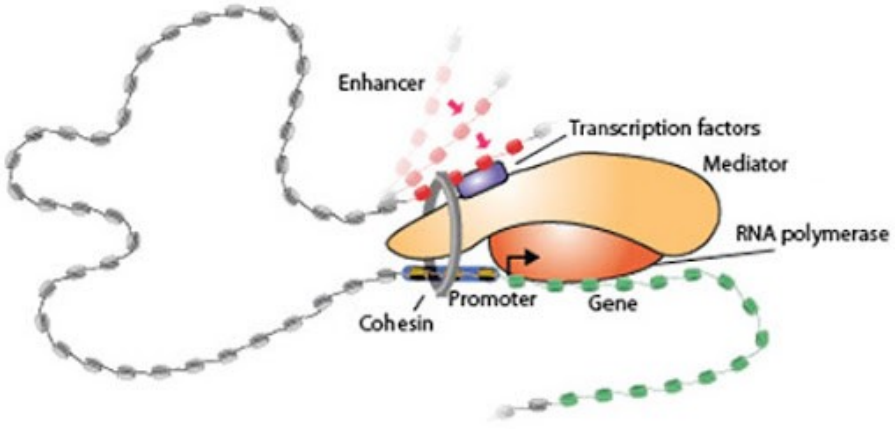

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Figure 6: Regulation of gene expression patterns by genomic enhancers.

DNA methylation can be induced by environmental factors that act on DNMTs or other elements influencing the DNA methylation machinery, such as the cofactor S-adenosylmethionine. There are numerous environmental "triggers" that have been associated with either global or site-specific DNA methylation alterations. The most widely studied class of environmental triggers of methylation are chemicals, including Aflatoxin B1, air pollution, arsenic, bisphenol-A, cadmium, chromium, lead, mercury, polycyclic aromatic hydrocarbons, persistent organic pollutants, and tobacco smoke. Other important determinants of DNA methylation modifications are nutritional factors. These nutrients are typically methyl donors such as methionine, folate, betaine, and choline (Martin and Fry, 2018). For example, higher levels of folic acid and vitamin B12 were correlated with increased ER gene methylation in patients with colorectal neoplasia (Al-Ghnaniem et al., 2007). Finally, non-chemical environmental factors, such as those related to the social environment, are increasingly shown to alter DNA methylation. These factors mainly include childhood adversities, and maternal mental health (e.g., depression) (Martin and Fry, 2018).

1.5.3 DNA methylation during aging: "epigenetic clock" and "epigenetic drift"

Apart from chemicals and non-chemical environmental factors, DNA methylation modifications have been associated with biological factors, including aging (Marttila et al., 2015). Several studies have demonstrated a decrease in blood DNA methylation in association with increasing age (Jones et al., 2015). These changes are more likely to occur at enhancers than promoters (Johansson et al., 2013). On the contrary, regions rich in CpG islands, such as promoters, tend to gain DNA methylation with age (Jones et al., 2015; Horvath et al., 2012).

Jones et al. (2015) distinguish two categories of age-dependent DNA methylation changes, described by the concepts of "epigenetic drift" and "epigenetic clock" (Figure 7). The concept of "epigenetic drift" points to modifications that occur due to the loss of regulatory control of DNA methylation mechanisms, which lead to increased variability of DNA methylation among aging individuals. According to Jones et al. (2015), epigenetic drift may be defined as a by-product of the aging process itself. In opposition, the epigenetic clock points to modifications leading to common DNA methylation changes across aging individuals. Among these common methylation changes, some are thought to constitute beneficial adaptive changes for the aging organism (Ashapkin et al., 2017; Avrahami et al., 2015; Ciccarone et al., 2018; Jones et al., 2015). Studies on mice showed that clock CpGs are mainly localized to promoters and enhancers (Field et al., 2018). For instance, the study of Avrahami et al. (2015) demonstrated age-associated hypomethylation of enhancers in mouse pancreatic β cells that led to better β-cell functioning in older mice. This study suggests that adaptive responses through DNA methylation changes may occur during aging through hypomethylation at enhancers (Ciccarone et al., 2018).

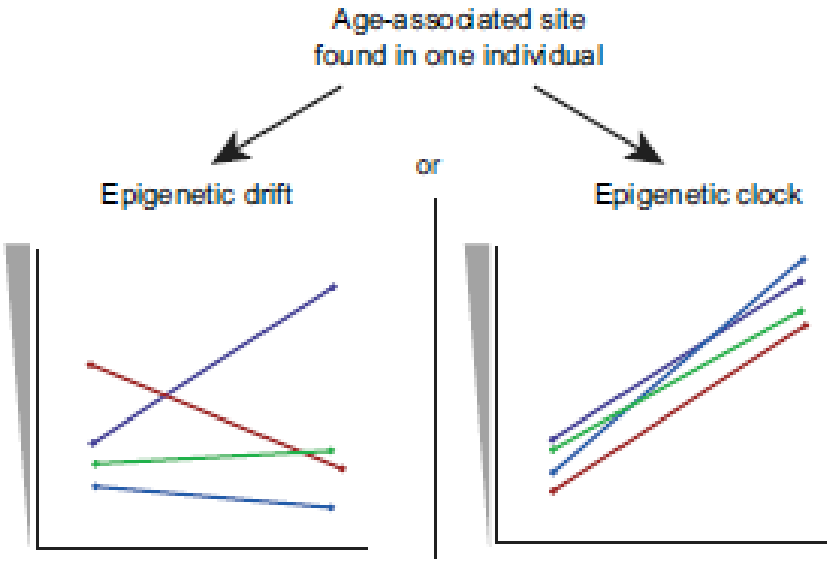

Figure 7: Epigenetic drift and epigenetic clock according to Jones et al., 2015. Epigenetic changes across aging individual can be inconsistent (epigenetic drift) or consistent (epigenetic clock).

Changes in levels of sex steroids have been described as potential determinants of the epigenetic clock (Jylhävä et al., 2017). From the perspective of evolution, this hormone-DNA methylation association would have positive effects on fitness-related during the fertile life, as the strength of natural selection decreases with age (Fabian and Flatt, 2011). Therefore, aging cannot be programmed. Instead, DNA methylation changes occurring during aging would be the continuations of developmental and reproductive programs that are not switched off after the fertile life. Evolutionists describe aging as "a purposeless quasi-program or, figuratively, a shadow of actual programs" (Blagosklonny, 2013). To further complicated things, the mechanisms by which natural selection would select DNA methylation marks during the fertile life have not been understood yet. Indeed, DNA methylation marks are eliminated during gametogenesis and early development, after fertilization (Flores et al., 2013; Morgan et al., 2005). Among other hypotheses, beneficial DNA methylation modifications at clock genes during the lifespan may be preserved across generations through the selection of genomic mechanisms associated with these methylation marks (Flores et al., 2013; Tikhodeyev, 2020).

1.5.4 Estrogen receptor genes: *ESR1*, *ESR2*, *GPER*

The three ERs, ERα, ERβ, and GPER, are encoded by separate genes located on different chromosomes (Cui et al., 2013).

The human ERα gene (*ESR1*) is a large genomic segment that spans ~300 kb and is located on chromosome 6q24. *ESR1* includes eight exons that encode the full-length 66 kDa protein that is composed of 595 amino-acids (Yaşar et al., 2017). The human *ESR1* is preceded by at least seven promoters, each associated with a CpG island, that can initiate transcription. In rats, promoter 0/B is equivalent to promoter C in humans, and mice (Wilson et al., 2008). Promoter C presents the highest percentage of sequence homology among the human, mouse, and rat (Figure 7). This indicates that promoter C has been maintained by natural selection through evolution, suggesting important biological function of this region (Orgel et al., 1968; Wilson

et al., 2008). The *ESR1* shore of promoter C has been described as an enhancer (ID GH06J151804, hg38-chr6: 151,804,757-151,805,897) of target promoters, including promoter A (Fishilevich et al., 2017; Tsuboi et al., 2017). In addition, transcription of this shore has been shown to dominate ERα expression in the whole brain of adult mice and in breast cells in humans (Tsuboi et al., 2017; Wilson et al., 2008). Importantly, DNA methylation of the *ESR1* shore of promoter C strongly decreases ERα expression and, as suggest by recent evidence, it may be positively regulated by E2 (Tsuboi et al., 2017; Ianov et al., 2017). Although the mechanism has not been clearly elucidated, the complex E2-ERα may upregulate repressors of methyltransferases, which in turn would increase methylation at the CpGs of promoter C (Ianov et al., 2017). This mechanism could contribute to the downregulation of ERα by E2, which is thought to maintain a balanced estrogen signaling (Ianov et al., 2017; Liu and Shi, 2015).

Similar to ERα, the human ERβ gene (*ESR2*), located on chromosome 14q22, is a large genome segment spanning 254 kb with eight encoding exons (Figure 8) (Yaşar et al., 2017). The ERβ protein is produced from eight exons and consists of 530 amino acids, with a molecular mass of 60 kDa. The transcription of the human ERβ gene occurs from at least two different promoters, named promoter 0N and promoter 0K. Promoter 0N has been cloned and proven to have promoter activity (Zhao et al., 2008), while promoter 0K has not been characterized in detail. However, both promoters exhibit CpG islands, and it is clear that ERβ expression can be regulated by DNA methylation in these CpG islands, at least in those around exon 0N (Svedenborg et al., 2009).

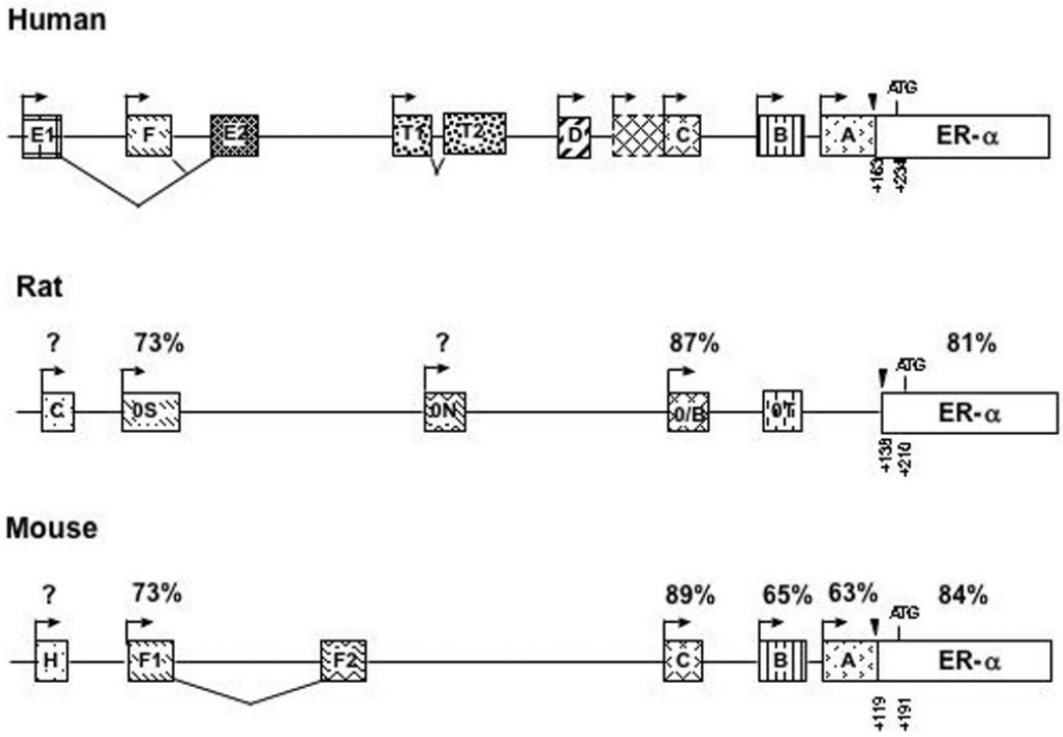

Figure 8: Schematic representation of the promoter regions of the human, rat and mouse *ESR1* (Wilson et al., 2008). The boxes represent promoters of the *ESR1*. The percentages above the ERα protein refer to the percent homology compared to humans.

Up to now, the human GPER gene (*GPER*) is the less well-characterized among the three ERs gene. *GPER* is located on chromosome 7p22 and spans 12 kb. Two non-coding exons (exon 1 and 2) and one coding exon (exon3) have been identified. The coding exon encodes a 375-amino acid protein. The one-known promoter of *GPER* partly overlap exon 1 and exon 2 (Figure 9) (Chevalier et al., 2014).

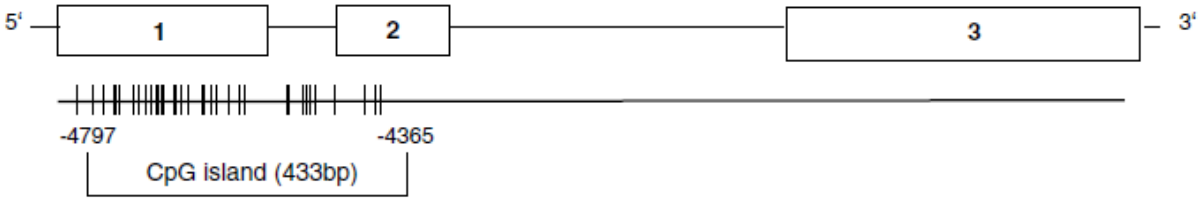

Figure 9: Schematic representation of genomic structure with the proximal promoter of *GPER* (Weissenborn et al., 2014).

1.6 Estrogen receptor genes methylation in health and disease

In the brain, increased methylation of *ESR1* may be linked to altered stress responses and behavioural changes (Wilson et al., 2008). Indeed, studies on rats showed that decreased expression of ERα in the POA, due to increased methylation of the 0/B promoter, leads to the inability of E2 to regulate oxytocin activity, which results in more pronounced stress responses (Champagne et al., 2006). The same study showed that along with changes in stress responses, increased methylation of the ERα 0/B promoter is also associated with the inheritance of maternal behaviour. Indeed, through DNA methylation, variations in the rates of licking and grooming are inherited such that mothers with high rates of licking and grooming activity have pups that show the same behavior when they become mothers. Clinical studies reported that in the ovarian tissue, hypermethylation of *ESR1* promoter B and C is associated with endometriosis (Maekawa et al., 2019). In the human colon, blood, and in the cardiovascular system, *ESR1* methylation of proximal promoter (A and B) increases with age and it is associated with colon neoplasia, atherosclerosis and risk of developing osteoporosis (Issa, 2002; Lv et al., 2011). In women with breast cancer, hypermethylation of the *ESR1* promoter region is associated with decreased ERα expression, shorter survival and endocrine treatment resistance (Fontes-Sousa et al., 2019; Gaudet et al., 2009; Kirn et al., 2018; Sheng et al., 2017; Tsuboi et al., 2017). Interestingly, increased methylation of *ESR1* promoter in blood appears to be a very useful biomarker for the diagnosis of breast and lung cancer (Khakpour et al., 2015; Suga et al., 2008). Similar to *ESR1*, increased methylation of *ESR2* promoter 0N was described in breast and ovary cancer, endometriosis, atherosclerosis, and vascular senescence (Bean et al., 2014: Swedenborg et al., 2009). Up to now, *GPER* promoter methylation has been only described in breast cancer. Hypermethylation of *GPER* promoter leading to decreased GPER expression is associated with worst survival in women patients (Weissenborn et al., 2017).

Apart from the work of Champagne et al. (2006), little is known concerning ERs promoter methylation in the brain. The links between ER genes methylation and neurological disorders are today still relatively unexplored (Wilson et al., 2008). One recent study demonstrated that *ESR1* promoter methylation in blood is associated with impaired cognitive function in patients with Alzheimer's disease (Li et al., 2018).

1.7 DNA methylation analysis

1.7.1 Sources of DNA

All tissue that has not been degraded can be used as a source of DNA (Deagle et al., 2006). Generally, the tissue associated with the phenotype of interest is the tissue of choice from which extracting DNA and studying DNA methylation patterns. However, some tissues can be difficult or even impossible to access. For example, methylation studies that target the human brain have to rely on post-mortem tissues, which has clear unpractical implications. Therefore, peripheral tissues are currently used as a surrogate for tissues of difficult access (Rhein et al., 2015). The most widely used peripheral tissues as a surrogate are blood, buccal cells, and saliva (Yoshizawa et al., 2013). Blood has been used as a proxy of other tissues in studies assessing DNA methylation of the ER genes. Some of these studies have found that differential DNA methylation of the ER genes in the blood was associated with age-related diseases that affect the functionality of other tissues (Khakpour et al., 2015; Li et al., 2018; Lv et al., 2011). For instance, increased methylation of the *ESR1* in blood was significantly associated with Alzheimer's diseases, which affects brain functions (Li et al., 2018).
In methylation studies, blood is generally collected from veins and capillaries. The dried blood spot (DBS) technology consists of sampling capillary whole blood and spot it on filter paper (Figure 10) (Fischer et al., 2019). This technology has previously been successfully used for evaluating DNA methylation in the context of methylation studies (Aberg et al., 2013). There are various advantages with DBS. Blood spots are generally collected from finger-pricks (or, for infants and young children, from heel-pricks). This relative non-invasive blood collection technique poses minimal risks to the participant and may therefore be of special interest for studies involving vulnerable individuals, such as elderly people, or patients suffering from vascular diseases. Other advantages of blood spots are that they are easy to storage, shipping and handling. For instance, blood collected in tubes (heparin or EDTA tubes) should ideally be stored at +4°C and processed as soon as possible to achieve optimum DNA yield and quality. In contrast, blood spots can be dried and shipped at room temperature. In addition, blood spots can be kept in a long-term storage facility (< -20°C) for years before the extraction of DNA without significant quality loss. Lastly, the collection procedure authorizes the collection to non-medical researchers and can be even self-administrated by adults without requiring the participants to visit a medical facility. This may be of particular interest in epidemiological studies where participants are asked to give blood samples at multiple time points and/or participants in the study are geographically widespread (Aberg et al., 2013; Fischer et al., 2019). The Qiagen-based protocol for DNA extraction from DBS adapted to the needs of the biochemical laboratory of the Psychology Institute of the University of Zurich (Appendix).

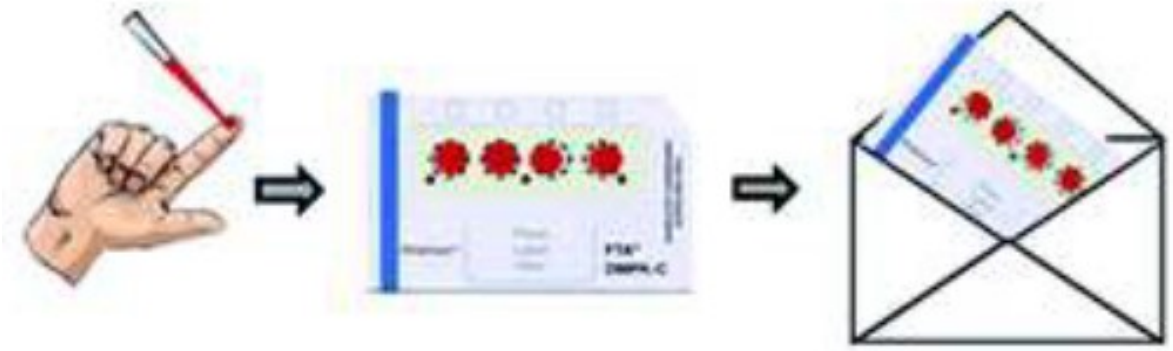

Figure 10: DBS sampling and shipping (Tretzel et al., 2015).

1.7.2 Tissue-specific methylation

Methylation is known to be cell- and tissue-specific. Therefore, the choice of the tissues can largely impact on results and their interpretation (Fiori and Turecki, 2016; Zhang et al., 2013). However, at some CpGs, DNA methylation variance is more closely linked to individual specificity than to tissue specificity, and some disease phenotypes affecting the brain or other organs are linked to differential methylation in peripheral tissues (Fiori and Turecki, 2016; Hannon et al., 2015). Hannon et al., (2015) developed a free online available tool to allow comparison of inter-individual methylation variance between the blood and four brain regions, using samples from 117 deceased individuals (both controls and individuals with variable levels of neuropathology).

1.7.3 Next-Generation Sequencing: library preparation and sequencing

Today, Next-Generation Sequencing (NGS) is widely used for studying DNA methylation patterns. After the completion of the human genome project in 2003, new technologies have been implemented to answer complex biological questions on the human genome that arose. To this end, various NGS technologies have been developed to allow higher throughput and lower costs of sequencing. High throughput refers to the capacity of sequencing multiple DNA molecules in parallel, enabling hundreds of millions of DNA molecules to be sequenced at a time (Churko et al., 2013). This allows discriminating small variations in the genome that may contribute to disease (Chen et al., 2017). The NGS instrument developed was the 454-pyrosequencing device. Pyrosequencing is a common sequencing approach, relatively simple and flexible. However, pyrosequencing provides good quality results only when short sequences (<200bp) are analysed (Shen and Qin, 2012). Good quality sequencing of longer DNA fragments is achievable using Illumina or Nextera technologies. However, chemical kits and services offered by these companies are expensive, which may be problematic for large-sample studies (Chen et al., 2017). Therefore, it is common for biochemical laboratories to developed customized NGS approaches based on Illumina and Nextera technologies in order to adapt the protocol to the specific needs of the lab and reduce the costs of the NGS procedure (Churko et al., 2013). Bisulfite-based arrays, such as the Infinium HumanMethylation450 (HM450) Beadchip are a very popular alternative to sequencing. However, this technology is limited to the analysis of preselected probes (Chen et al., 2017). For instance, the HM450 Beadchip allows the analysis of only a few CpGs of the ER gene promoters.

Recently, a customized NGS approach developed by the McGill Group for Suicide Studies (MGSS, McGill University) has been modified by the Biochemical laboratory of the Institute of Psychology in collaboration with the genomic diversity center (GDC, ETH Zurich). The procedure is divided into four main steps: 1) bisulfite treatment of genomic DNA, 2) library preparation, 3) sequencing, and 4) interrogation of GpGs in the sequencing products (Appendix). In the following paragraphs, general and complementary information on the procedure are described.

The bisulfite conversion of the DNA is the current gold standard for studying methylation modifications of the genome (Kint et al., 2018). The bisulfite treatment of the DNA converts unmethylated cytosines into uracil, which is subsequently substituted by thymine in the Polymerase Chain Reaction (PCR) during the library preparation. Instead, methylated cytosines are not affected by the bisulfite treatment (Figure 11) (Peedicayil, 2014). The alignment of the sequencing products to the reference DNA sequence allows distinguishing between methylated and unmethylated cytosines. The library preparation includes amplification of the targeted sequence, the addition of single DNA barcodes for indexing samples, and the pooling of amplicons if more than one amplicon are sequenced in the same sequencing run. The major modifications of the original procedure are the elimination of the first amplification step and the use of E-gel size selection (Thermo Fisher Scientific) for the purification of the PCR products. The E-gels technology is highly efficient, as it allows the complete purification of PCR products while conserving the same amount of the amplicon. This eliminates the need for multiple amplification/purification steps during the library preparation, which instead, are necessary when using the AMPure beads technology as a purification approach. In addition, compared to AMPure beads, the purification with e-gels is a much easier task.

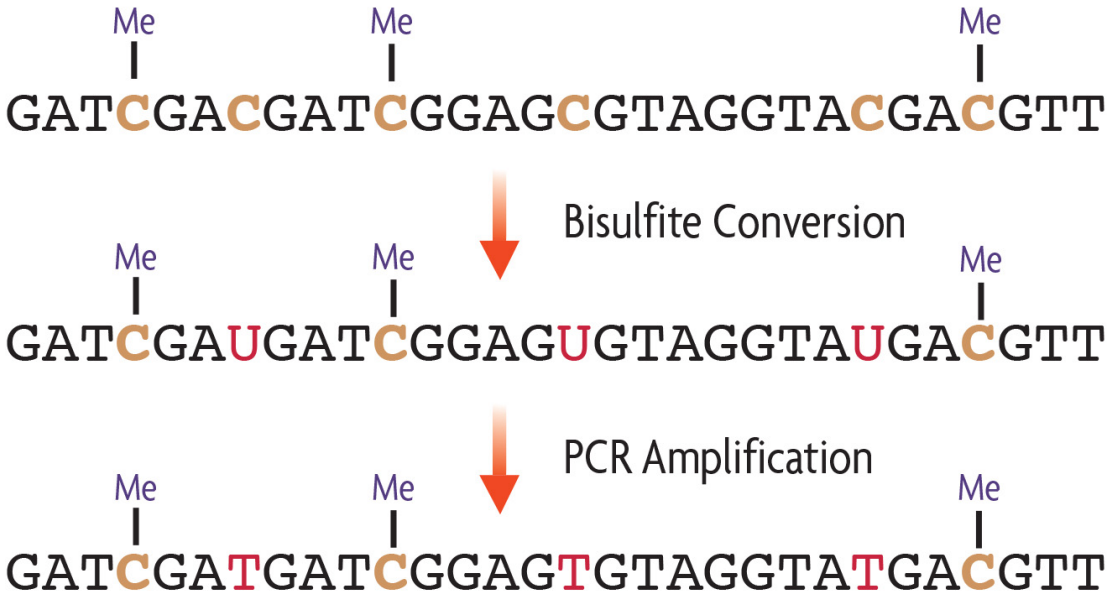

Figure 11: Schematic illustration of bisulfite conversion (Actif Motif, 2020).

Before starting the library preparation, specific primers for the targeted amplicons need to be designed. This requires the following steps:

1) Identification of the sequence of interest on a genome browser, such as UCSC (https://genome.ucsc.edu/).
2) Virtual bisulfite conversion of the DNA sequence using a bisulfite conversion program, such as MethPrimer (https://www.urogene.org/methprimer/).
3) For each targeted amplicon, designing the forward and reverse primers following the following rules: a) primer length between 19-24 bp with a Tm of 60°C and a Tm difference between forward and reverse primer of 1-2 °C, b) distance between the forward and reverse primer of max 500 bp, c) primers do not have to contain CG, while the content of C should be maintained low (1 or 2 C in the primer),d) the forward primer (5'->3') is a copy of the corresponding portion of the DNA, while the reverse primer is obtained by reversing the orientation of the corresponding portion of the DNA and substituting each nucleotide with the complementary one.
4) Primer ordering from a supplier of custom nucleic acids, such as IDT (https://eu.idtdna.com/pages). The primers are ordered with the universal primers CS1 (forward primer) and CS2 (reverse primer) on 5' position, separated from the specific primer by three random nucleotides.
5) Optimization of the cycling conditions to optimize the yield of the targeted product and minimize PCR artifacts, such as non-specific amplification or amplification of primers (primer-dimers). If the amplification test fails or does not work properly, it is possible to vary the following parameters: primer concentration (typically in the range 10 to 0.5 µM), annealing temperature (typically, temperatures range from 50 to 64°C), number of amplification cycles (up to 60 cycles), amount of starting DNA (up to 3 ng). If, after varying these parameters the PCR still fails to correctly amplify the desired DNA sequence, new primers need to be designed.

The sequencing is performed on an Illumina Miseq instrument, which is available at the GDC upon registration. The interrogation of CpGs in the sequencing products is performed using specific software, and require the expertise of a bioinformatician.

1.8 Genetic polymorphisms of the ER genes and women health

Genetic variants or polymorphisms of the ER genes are other factors that have been associated with women's health. The literature on *ESR1* polymorphisms is dominated by two SNPs: the *PvuII* polymorphism (rs2234693) and the *XbaI* polymorphism (rs9340799), located in an intron between exons 1 and 2. These are known as "restriction fragment length polymorphisms" because they disrupt sites at which the restriction enzymes, PvuII or Xbal, cut the DNA. The *PvuII* and *XbaI* polymorphisms were first associated with breast cancer and loss of bone density. Associations with many behavioral phenotypes have now been discovered, for example with the expression of anger and with episodic memory in women. These polymorphisms are also associated with clinical outcomes such as anxiety, depression in women, obsessive-compulsive disorder, and Alzheimer's disease (Lee and Song, 2015; Maney, 2017; Sundermann et al., 2010). The third most popular *ESR1* polymorphism consists of a dinucleotide (TA) repeat upstream of exon 1. The number of repeats ranges between nine and 27 and is bimodally distributed, with frequency peaks at 14 and 23 repeats. This tandem repeat has been linked with aggression in men, harm avoidance, and non-conformism in women as well as to clinical outcomes such as psychoticism in women, anxiety, conduct disorder, postpartum depression, cardiovascular diseases, and osteoporosis (Comings et al.,

1999; Mondockova et al., 2018; Pinsonneault et al., 2013; Sumi et al., 2019; Westberg et al., 2003).

The *ESR1* polymorphisms *PvuII*, *XbaI*, and the TA repeat are close together and in linkage disequilibrium (Figure 12). In linkage disequilibrium, some combinations of alleles occur more frequently in a population than would be expected from random assortment. Therefore, because the TA repeat, *PvuII*, and *XbaI* are in linkage disequilibrium, one can predict the allele of one genetic variant given the allele of another genetic variant with a high level of confidence. The T and C allele of the *PvuII* are commonly referred to as the p and P allele, respectively. The A and G alleles of *XbaI* are commonly referred to as the x and X alleles, respectively. The X and P are the variant alleles that lack the respective XbaI and PvuII restriction enzyme recognition sites. The risk alleles, low numbers of TA repeat, the p allele of *PvuII*, and the x allele of *XbaI* are likely to occur together (D9 = 0.755; p<0.000001) (Albagha et al., 2005; Sundermann et al., 2010). *ESR2* polymorphisms concentrated on the 3' end of the gene, downstream of exon 6 have been associated with clinical outcomes such as anorexia nervosa, autistic traits, cognitive impairment, and depression (Maney et al., 2017). Other SNPs have been associated with breast cancer (rs4986938) and osteoporosis (rs1256044) (Kaminski et al., 2018; Yu et al., 2011). The short allele of a CA (19–35) repeats (rs113770630), has been associated with osteoporosis and breast cancer, as well as reproductive disorders (Dalgleish et al., 2018; Lamp et al., 2011; Scariano et al., 2004; Zheng et al., 2012). For *GPER*, 40 single nucleotide polymorphisms have been identified so far. Of them, the P16L *GPER* variant is the most common with an allele frequency of approximately 20%. This polymorphism was associated with resistant hypertension in women and breast cancer (Feldman, 2016; Pupo et al., 2017).

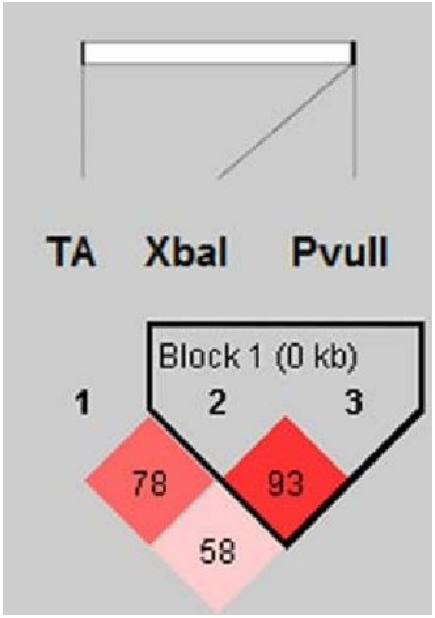

Figure 12: Linkage disequilibrium between TA, *PvuII* and *XbaI* polymorphisms in *ESR1* (Ayvaz et al., 2009).

Although associations between genetic variants and disease have been established, there is no definitive evidence concerning the functionality of these polymorphisms or the biological

pathways they affect (Sundermann et al., 2010). It is possible that, depending on their location on the gene, ER gene polymorphisms affect gene expression or protein structure, through mechanisms such as alternative splicing or epigenetic modifications (Wang et al., 2019). However, the direct association of the ER genes polymorphisms with gene expression has never been clearly demonstrated (Maney et al., 2017; Quilez et al., 2016).

1.9 Summary

The maintenance of estrogen signaling plays an important role in women healthy aging. In particular, the estrogen signaling in the hypothalamus contributes to preserving the physiological functions of a majority of systems in the body. The ERα is the main mediator of estrogenic effects in the hypothalamus, and in specific regions, such as the SCN, the distribution patterns of ERα are sexually dimorphic. In women, higher levels of this receptor are thought to compensate for the loss of E2 levels associated with menopause, while decreased levels would increase age-related disorders linked to E2 deficiency. In particular, as the SCN activities are importantly regulated by the ERα in women, decreased ERα expression in the SCN would alter vital functions such as circadian rhythms, which impact on the overall health. In the last decades, GPER has emerged as a potential mediator of the rapid effects of E2. Although little is known about GPER, several non-genomic effects of E2 formerly attributed to ERα have been suggested to be GPER-mediated. The contribution of ERβ in women healthy aging is mainly related to the prevention of cancer, obesity, and cardiac fibrosis. Aging is linked to DNA methylation modifications. Some of these modifications have been shown to improve physiological functions that are negatively affected by age, while others have been associated with age-related diseases. DNA methylation has been established as a key regulator of ERs expression during aging, and as a potential underlying mechanism of the E2 negative feedback on ERα expression in breast cells and in the rat hypothalamus. Genetic polymorphisms have been associated with various disorders, including some age-related diseases. However, a direct link between genetic polymorphism and ERs expression has never been described. Mechanisms such as DNA methylation and alternative splicing could possibly underly the association between ERs genetic variants and gene expression. Today, the development of new technologies, such as next-generation sequencing, make it possible to conduct association studies of DNA methylation at affordable costs, and to gain information on complex health-related biological questions. In addition, the growing use of peripheral tissues as surrogates allows a better understanding of the role of DNA methylation in body parts difficult to access, such as the brain. The most widely used peripheral tissues as a surrogate are blood, buccal cells, and saliva. Blood has been successfully used as a proxy of other tissues in studies assessing DNA methylation of the ER genes. The dried blood spot (DBS) technology consists of sampling capillary whole blood and spot it on filter paper, and it permits to accurately measuring DNA methylation. Unlike peripheral blood collected in tubes, blood spots can be shipped at room temperature, and stored at $< -20°C$ for several years before DNA extraction.

2. Conclusion, Aim of the study, and Research questions

Overall, the literature points to a key role of ERα in preserving the general health of women during aging, and to an important contribution of DNA methylation in modulating ERα expression. As various non-genomic effects of E2 could be GPER-mediated, methylation of the *GPER* promoter should also be considered in methylation studies on women healthy aging. DBS is the optimal technique for studies assessing ER genes methylation in healthy older adults, as it is minimally invasive, does not require medical expertise, and blood can be informative on the status of ER genes methylation in other tissues. Finally, DBS can be combined with bisulfite-NGS, which is the method of choice for novel methylation-association studies, as it allows to assess methylation at non-preselected CpGs within a defined DNA region. A better understanding of the role of ER genes methylation in women's health, and on how DNA methylation is modulated in healthy aging women could have important clinical implications, including the development of new therapies targeting DNA methylation.
As part of the Women 40+ Healthy Aging Study (Fiacco et al., 2019; Gardini et al., 2020; Mernone et al., 2019), the aim of this thesis was to assess ER genes methylation in 130 healthy Caucasian pre-, peri- and postmenopausal women aged 40–73 years to identify potential mechanisms underlying healthy aging. To achieve this aim, two studies have been conducted.

Study I: Differential *ESR1* Promoter Methylation in the Peripheral Blood—Findings from the Women 40+ Healthy Aging Study.

This study addressed the following research question: Do levels of methylation of the *ESR1* promoter A, *ESR1* promoter B, and the *ESR1* shore of promoter C differ among healthy middle-aged and older women with respect to the menopausal status, age, and E2 levels?

To answer this research question, the aims of this study were as follows:

a) To determine levels of methylation of the *ESR1* promoter A, *ESR1* promoter B, and the *ESR1* shore of promoter C from DBS, and to identify methylation changes in these regions related to age and menopausal status.
b) To determine levels of salivary E2 and analyse their association with methylation levels of *ESR1* promoter A, *ESR1* promoter B, and *ESR1* shore of promoter C.

To address these aims, DNA methylation was quantified using targeted bisulfite-NGS, while salivary E2 was quantified using enzyme-linked immunosorbent assay (ELISA).

Study II: Sleep and Methylation of Estrogen Receptor Genes, *ESR1* and *GPER*, in Healthy Middle-Aged and Older Women: Findings from the Women 40+ Healthy Aging Study

This study addressed the following research question: Are levels of *ESR1* and *GPER* methylation associated with phenotypes related to dysfunctional estrogen signaling in the hypothalamus, specifically, sleep problems and vasomotor symptoms?

To answer this research question, the aims were as follows:

a) To determine levels of methylation of the *GPER* promoter from DBS, and collect data on sleep problems and VMS.
b) To conduct a series of path analyses to determine whether levels of *ESR1* and *GPER* methylation were associated with increased sleep problems, either directly, or indirectly through VMS.

To address this aim, DNA methylation was quantified using targeted bisulfite-NGS, while sleep and VMS were assessed using the Pittsburgh Sleep Quality Index (PSQI) and the one item ("hot flashes/sweating") of the somato-vegetative subscale of the Menopause Rating Scale (MRS) respectively.

3. Results

3.1 Study I: Differential *ESR1* Promoter Methylation in the Peripheral Blood—Findings from the Women 40+ Healthy Aging Study

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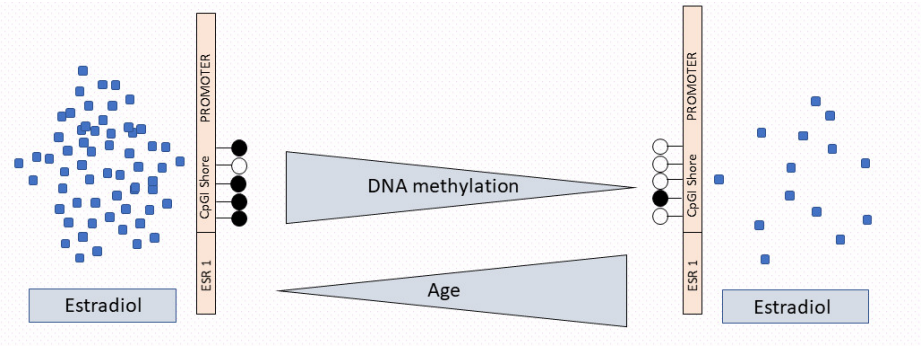
Background Estrogen receptor α (ERα) contributes to maintaining biological processes preserving health during aging. DNA methylation changes of ERα gene (*ESR1*) were established as playing a direct role in the regulation of ERα levels. In this study, we hypothesized decreased DNA methylation of *ESR1* associated with postmenopause, lower estradiol (E2) levels, and increased age among healthy middle-aged and older women. **Methods** We assessed DNA methylation of *ESR1* promoter region from dried blood spots (DBSs) and E2 from saliva samples in 130 healthy women aged 40–73 years. **Results** We found that postmenopause and lower E2 levels were associated with lower DNA methylation of a distal regulatory region, but not with DNA methylation of proximal promoters. **Conclusion** Our results indicate that decreased methylation of *ESR1* cytosine-phosphate-guanine island (CpGI) shore may be associated with conditions of lower E2 in older healthy women.

Keywords: *ESR1* promoter; DNA methylation; CpGI shore; estradiol; women healthy aging



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3.1.1 Introduction

Human life expectancy has been growing at a rapid rate (WHO, 2020), but this prolongation of life has not been accompanied by a proportional increase in the quality of life (Brown, 2015). Indeed, along with life expectancy, the incidence of age-related disabilities and comorbidities is also increasing (Brown, 2015). Women are more likely to survive into older ages than men and are therefore also exposed to a higher risk of age-related disabilities Ostan et al., 2016). As such, it is becoming increasingly important to identify factors which influence the physical aging process and extend healthy aging, especially in women (WHO, 2007).

Levels of ovarian hormones have been proposed as important factors influencing health among older women (Horstman et al., 2012; Fiacco et al., 2018). Estradiol (E2), the most potent form of estrogen, has important biological functions in addition to those associated with reproduction. These include regulatory functions of the cardiovascular system, central nervous system, skeletal homeostasis, and lipid and carbohydrate metabolism (Vrtačnik et al., 2014). In premenopausal women, the ovaries are the principal source of E2 (Simpson, 2003). However, with the onset of menopause, the ovaries' production of E2 progressively ceases (Santoro, 2005). Therefore, E2 levels decrease as women reach perimenopause, and enter into a low, steady level as women reach postmenopause (Gholizadeh et al., 2018). Declining E2 levels during perimenopause and low E2 levels during postmenopause appear to contribute to the increased incidence of diseases in older women, such as metabolic diseases (Lizcano, and Guzmán, 2014; Karvonen-Gutierrez et al., 2016), cognitive decline (Luine, 2014), mental disorders (Llaneza et al., 2012; Riecher-Rössler and Kulkarni, 2010), osteoporosis, and cardiovascular diseases (Eastell et al., 2016; Stevenson et al., 2011) . However, evidence indicates that low levels of estrogen receptors (ERs) may also play a role in the exacerbation of age-related diseases (Deroo and Korach, 2006; Iorga et al., 2017; Mott and Pak, 2013; Wynne et al., 2004).

ERs mediate the effects of estrogen through genomic and non-genomic mechanisms across a wide range of cells and tissues (Liu and Shi, 2015; Paterni et 2014). Genomic effects, which occur over several hours, are mediated by ERα and ERβ, also known as classical ERs (Paterni et al., 2014). Following activation by estrogen binding, these ERs translocate from the cell cytoplasm to the cell nucleus, where they contribute to the transcriptional activity of an important number of downstream genes (Heldring et al., 2007; Piva et al., 1992). On the cell plasma membrane, ERα, ERβ, and the more recently described G protein-coupled estrogen receptor (GPER) mediate rapid, non-genomic estrogenic effects (Girgert et al., 2019).

Studies from humans and rodent models indicate that, among the three ERs, ERα may be a key player in preserving health in advanced age. Indeed, ERα contributes to maintain biological functions such as cardiovascular, metabolic, cognitive, hypothalamic, and limbic functions, even under conditions of low estrogen levels (Bean et al., 2014; Gouw et al., 2017). This might be due to the higher affinity of ERα with E2, compared to the affinity with E2 of the other ER subtypes (Foster, 2012; Luo and Liu, 2020). Moreover, studies on osteoporosis indicate that ERα, but not ERβ, is essential in promoting bone-protective actions and bone formation (Deroo and Korach, 2006; Hertrampf et al., 2008). Studies have also reported a greater role of ERα compared to ERβ in protecting cardiovascular functions (Deroo and

Korach, 2006). Furthermore, a decrease in the relative expression of ERα/ERβ, mainly due to a loss of ERα, is associated with cognitive impairments and a loss of E2 responsiveness in advanced age (Bean et al., 2014; Foster, 2012). Concerning GPER, its role as a plasma membrane-based ER is controversial, and there is still a lack of evidence that this ER plays a significant role in mediating endogenous estrogen effects in vivo (Luo and Liu, 2020).

Levels of ERα are regulated, at least in part, by mRNA expression (Ianov et al., 2017). DNA methylation is a key epigenetic mechanism, which regulates mRNA expression through the binding of methyl groups at cytosines in cytosine-guanine dinucleotides (CpGs) (Kumar et al., 2018). These DNA modifications are influenced by various internal and external environmental factors and occur without altering the underlying DNA sequence (Mohtat and Susztak, 2010). Generally, hypermethylation of transcriptional regulatory regions is associated with gene silencing, while hypomethylation is associated with gene activation, resulting in increased mRNA expression (Baribault et al., 2018). DNA methylation changes have been established as playing a direct role in the transcriptional regulation of ERα gene (*ESR1*) (Pinzone et al., 2004).

Aging is associated with DNA methylation modifications. Jones et al. (2015) distinguished two categories of age-dependent DNA methylation changes, illustrated by the concepts of "epigenetic drift" and "epigenetic clock". "Epigenetic drift" refers to modifications that occur due to the loss of regulatory control of DNA methylation mechanisms, and result in increased variability of DNA methylation across aging individuals. By contrast, "epigenetic clock" refers to modifications leading to common DNA methylation changes across aging individuals. Among the common methylation modifications, some may constitute beneficial adaptive changes (Ashapkin et al., 2017; Avrahami et al., 2015; Ciccarone et al., 2018; Jones 2015). These adaptive changes may be the product of natural selection (Flores et al., 2013; Tikhodeyev , 2020). However, associations between DNA methylation and adaptive evolution have not been clearly elucidated. Indeed, DNA methylation marks contribute to adaptive phenotypic variation, but, in mammals, they are erased during early development, following fertilization (Flores et al., 2013). Among other hypotheses, beneficial environmentally induced methylation profiles (i.e., changes promoting reproductive functions and longevity) may be maintained across generations through the selection of genomic mechanisms linked to these methylation profiles (Flores et al., 2013; Tikhodeyev, 2020).

A recent epigenome-wide analysis may constitute an example of beneficial age-dependent DNA methylation changes (Avrahami et al., 2015). Indeed, age-associated DNA hypomethylation of distal regulatory elements (enhancers) was related to the upregulation of genes essential for cell identity and function. As a consequence, these DNA methylation changes promoted better β-cell function in older mice, suggesting that adaptive responses through DNA methylation changes may occur during aging (Avrahami et al., 2015; Ciccarone et al., 2018). Regarding *ESR1*, recent evidence suggests that methylation of its promoter may be modifiable across the life span, acting as a regulatory mechanism for ERα expression (Ianov et al., 2017; Schwarz et al., 2010; Tsuboi et al., 2017). For instance, Ianov et al. (2017) showed that altered methylation of specific CpGs of *ESR1* promoter was associated with age, ovariectomy, and ERα expression in the hippocampus of female rats. To the best of our knowledge, no study has yet investigated potential *ESR1* methylation patterns associated with women's aging. Changes of *ESR1* promoter methylation may contribute to maintaining

health among older women. Therefore, in this study, we hypothesized that lower levels of methylation at three DNA regions of the *ESR1* promoter (proximal promoter A and B, and CpG island shore) would be associated with postmenopause, lower E2 levels, and increased age among healthy women. Our results indicate alterations of *ESR1* cytosine-phosphate-guanine island (CpGI) shore methylation in aging women, and may provide new insights for further investigations in the field of the female health span.

3.1.2 Materials and Methods

Subjects

Women aged 40–75 years were recruited in the context of the Women 40+ Healthy Aging Study, a larger cross-sectional investigation including healthy middle-aged and older women (Mernone et al., 2019, Fiacco et al., 2019; Fiacco et al., 2020). To be included in the study, women had to report good, very good, or excellent health. Women were excluded from the study if they met at least one of the following criteria: acute or chronic somatic disease; acute or chronic mental disorder; psychotherapeutic treatment and use of psychotropic drugs during the last six months; more than two standard units of alcoholic beverages per day; pregnancy in the last six months; menopause due to surgical removal of the ovaries or the uterus; precocious menopause; current use of oral contraceptives or use of hormone therapy; disease of the thyroid gland, pancreas, adrenal gland or ovaries influencing the endocrine system; diabetes, polycystic ovary syndrome (POCS), hirsutism, endometriosis, and hyper- or hypothyroidism. Subjects were divided into three subgroups, with respect to their menopausal status according to the Stages of Reproductive Aging Workshop +10 (STRAW) criteria: (1) premenopausal, if the menstrual cycle was regular, (2) perimenopausal, if the cycle length was variable, with variability among cycles of at least seven days, or if the interval between cycles was > 60 days, and (3) postmenopausal if no bleeding had occurred in at least the last 12 months (Harlow et al., 2012). All subjects gave their informed consent for inclusion before they participated in the study. The study (BASEC Nr 2016-01591) was conducted in accordance with the Declaration of Helsinki, and approved on 2 December 2016 by the Cantonal Ethics Committee of the canton of Zurich (KEK Zurich, Zurich, Switzerland).

Biological Sampling

Saliva and peripheral blood samples were collected at 8:00 am under standardized conditions. In pre- and perimenopausal women, sampling was conducted in the early follicular phase, during which E2 levels are low (Reed et al., 2018). One or two drops of blood were collected from fingertips onto S&S 903 Whatman® paper cards (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blood spots were dried at room temperature for about 3 h and stored at −20 °C until subsequent DNA extraction. Participants were asked to collect saliva into 2-ml SaliCaps (IBL International GmbH, Hamburg, Germany) using the passive drool method. Saliva samples were stored at −20 °C until biochemical analysis.

Methylation Analysis

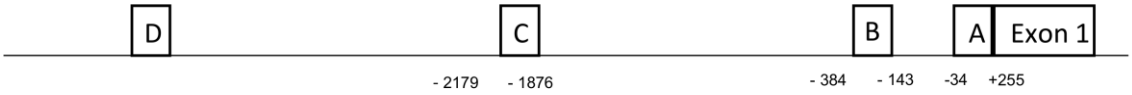
DNA isolation—We used the dried blood spot (DBS) technology as source of genomic DNA. Regulation of ERα expression by E2 has been demonstrated in the blood (Kim et al., 2004).

Therefore, it is possible that DNA methylation changes underlying the ERα regulation by E2 occur in the blood. The use of DBS technology has practical implications in terms of tissue accessibility and storage and has previously been successfully used for evaluating cytosine methylation (Aberg et al., 2013; Fischer et al., 2019). Genomic DNA was extracted from three punches of 3 mm diameter using theQIAamp DNA Investigator Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions, and eluted in a final volume of 30 µL of RNase-free water. Qubit (Thermo Fischer Scientific, Waltham, MA, USA) was used to assess the DNA concentration.

Bisulfite conversion—Genomic DNA (41–168 ng) was bisulfite-treated using the EZ 96-DNA methylation-Gold kit (Zymo Research, Irvine, CA, USA). The manufacturer's instructions recommend using samples containing 0.5–2000 ng of DNA. Bisulfite converted DNA was eluted in 20 µL of RNase-free water and stored at −80 °C until subsequent analysis.

NGS Library preparation—We analyzed three DNA sequences located in the promoter region of *ESR1*. Two sequences are located in two CpGI, one in proximal promoter A and one in proximal promoter B. The third sequence is a CpGI shore near promoter C, located approximately 2 kbp upstream of promoter A (Figure 1) (Tsuboi et al., 2017). Increased methylation at these regulatory DNA sequences has been shown to decrease *ESR1* expression and to be associated with diseases (Gaudet et al., 2009; Issa et al., 1994; Kirn et al., 2018; Post et al., 1999; Sheng et al., 2017). The PCR amplicon library preparation for next-generation bisulfite sequencing was based on the protocol described by Chen et al. (2017). An initial polymerase chain reaction (PCR) was performed on the bisulfite-treated DNA using the Kapa HIFI Uracil+ master mix (Kapa Biosystems, Wilmington, MA, USA). Bisulfite primers were designed manually or using MethPrimers (Li and Dahiya, 2002). Primers contained universal oligonucleotides CS1/CS2 (Fluidigm, San Francisco, CA, USA, Table 1), used for customized NGS sequencing primers. PCR conditions were 95 °C for 3 min, then 40 cycles of 98 °C for 20 s, 54–60 °C for 15 s, 72 °C for 15 s, and a final step with 72 °C for 45 s. PCR amplicon products were purified using E-gels 2% size selection (Thermo Fisher Scientific, Waltham, MA, USA). To verify that primers were specifically amplifying bisulfite converted DNA, positive and negative controls (bisulfite converted DNA and genomic DNA, respectively) were included in the PCR. Then, a second PCR of 10 cycles (Tm 60 °C) was performed for adding the Illumina NGS library flowcell attachment sites and customized single barcode for each individual (Fluidigm, San Francisco, CA, USA). A final purification of the pooled amplicon libraries from each of the three DNA regions (promoter A, B, and CpGI shore) was performed, and final products were quantified using the Agilent 2200 Tape Station instrument and HS DNA 1000 reagents (Agilent Scientific Instruments, Santa Clara, CA, USA). The three DNA sequences were pooled at a final molarity of 2 nM. To increase the diversity of base calling during sequencing we added PhiX spike-in (12%) to the library. The final library was sequenced on the Illumina MiSeq using the V3, 600 cycles kit (300 PE) (Illumina, San Diego, CA, USA).

A



B

GCTCCCTGTGAGCAGACAGCAAGTCTCCCCTCACTCCCCACTGCCATTCATCCAG[1]CGCTGTGCAGTAGCCCA
GCTG[2]CGTGTCTGC[3]CGGGAGGGGCTGCCAAGTGCCCTGCCTACTGGCTGCTTCC[4]CGAATCCCTGCCATTCC
A[5]CGCACAAACACATCCACACACTCTCTCTGCCTAGTTCACACACTGAGCCACT[6]CGCACATG[7]CGAGCACATTC
CTTCCTTCCTTCTCACTCTCT[8]CGGCCCTTGACTTCTACAAGCCCATGGAACATTTCTGGAAAGA[9]CGTTCTTGA
TCCAGCAGGGTAGGCTT

C

GGGGAATCAAACAGAAAGAGAGACAAACAGAGATATAT[1]CGGAGTCTGGCA[2]CGGGGCACATAAGGCAGCACA
TTAGAGAAAGC[3]CGGCCCCTGGATC[4]CGTCTTT[5]CG[6]CGTTTATTTTAAGCCCAGTCTTCCCTGGGCCACCTTTA
GCAGATCCT[7]CGTG[8]CGCCCC[9]CGCCCCCTGGC[10]CGTGAAACTCAGCCTCTATCCAGCAG[11]CGA[12]CGACAAGT
AAAGTAAAGTTCAGGGAAGCTGCTCTTTGG

D

AGACCAGTACTTAAAGTTGGAGGCC[1]CGGGAGCCCAGGAGCTGG[2]CGGAGGG[3]CGTT[4]CGTCCTGGGACTGCA
CTTGCTCC[5]CGT[6]CGGGT[7]CGCC[8]CGGCTTCAC[9]CGGACC[10]CGCAGGCTCC[11]CGGGGCAGGGC[12]CGGGGCCA
GAGCT[13]CG[14]CGTGT[15]CGG[16]CGGGACATG[17]CGCTG[18]CGT[19]CGCCTCTAACCT[20]CGGGCTGTGCTCTTTTTCCA
GGTGGCC[21]CGC[22]CGGTTTCTGAGCCTTCTGCCCTG[23]CGGGGACA[24]CGGTCTGCACCCTGCC[25]CG[26]CGGCCA
[27]CGGACCATGACCATGACCCTCCACAC

Figure 1: (A) Schematic figure of *ESR1* promoter region. (B) Assessed DNA sequence (−2179; −876) in CpGI shore near promoter C, including nine CpGs. Methylation at CpGs 1–9 was associated with E2 deprivation in humans (Tsuboi et al., 2017). Altered methylation at these CpGs region was associated with ovariectomy and age in the hippocampus of female rats (Ianov et al., 2018). (C) Assessed DNA sequence (−384; −143) in promoter B, including 12 CpGs located in a CpGI. (D) Assessed DNA sequence in Promoter A (−34; +255), including 27 CpGs located in a CpGI. Underlined sequences correspond to the primers position.

Table 1: Primers used for assessing DNA methylation in the promoter regions of *ESR1*.

Target	Forward primer	Reverse primer	GRC h37 (hg19)	T (°C)
CpGI shore	ACACTGACGACATGGTTCTACA NNN GTTTTTTGTGAGTAGATAGTAAGTT	TACGGTAGCAGAGACTTGGTCT NNN AAACCTACCCTACTAAATCAAAAAC	chr6: 152126660-152126963	58
Promoter B	ACACTGACGACATGGTTCTACA NNN GGGGAATTAAATAGAAAGAGAGATAAATAG	TACGGTAGCAGAGACTTGGTCT NNN CCAAAAAACAACTTCCCTAAACTT	chr6: 152128433-152128671	60
Promoter A	ACACTGACGACATGGTTCTACA NNN AGATTAGTATTTAAAGTTGGAGGTT	TACGGTAGCAGAGACTTGGTCT NNN ATATAAAAAATCATAATCATAATCC	chr6: 152128780-152129067	54

Note: forward primers include the universal Fluidigm primer sequence CS1 (ACACTGACGACATGGTTCTACA), while reverse primers include the universal Fluidigm primer sequence CS2 (TACGGTAGCAGAGACTTGGTCT). NNN between universal primers CS1/CS2 and bisulfite primers represent randomized nucleotides to molecular diversity generation during sequencing (O'Donnell et al., 2016). Abbreviations: CpGI = cytosine-phosphate-guanine island.

Interrogation of CpGs in the targeted amplicons—Adaptor sequences and low-quality bases were removed using the default settings of trimmomatic v0.35 (licensed under GPL V3 and available at http://www.usadellab.org/cms/index.php?page=trimmomatic) (Bolger et al., 2014). Only paired-end sequences > 2 × 20 bp were kept, which were then aligned to the target regions, and counts were extracted using Bismark program (v0.19.0). A customized R script was subsequently used to parse all counts. In accordance with Chen et al. (2017), a minimum threshold of 100× reads was set. The number of total reads across the samples ranged from 119 to 31,598 (M = 6227, SD = 5240) for promoter A, from 202 to 63,844 (M =

3219, SD = 10,946) for promoter B, and from 148 to 48,993 (M = 19,512, SD = 13,972) for CpGI shore. Finally, unconverted CpGs percentage was calculated for each CpG as the number of unconverted reads divided by the total read count. Levels of methylation were consistent with previous data in blood (retrieved from the GSE40279 dataset) (Hannum et al., 2013). Methylation was low in promoters A and B, while the CpGI shore had intermediate levels of methylation (Table 2). Methylation data are available in "Dryad" at https://doi.org/10.5061/dryad.gmsbcc2jK.

Estradiol Measurement

E2 levels were determined using the 17beta-Estradiol Saliva Luminescence Immunoassay (IBL International, Hamburg, Germany). Intra- and inter-assay coefficients were below 13.3% and 14.8%, respectively, and the assay's analytical sensitivity (limit of detection) was 1.1 pmol/L. According to the manufacturer instructions, E2 values range between 2.9 and 28.2 pmol/L during the follicular phase, while in postmenopausal women expected values are lower than 15.7 pmol/L (IBL International, Hamburg, Germany, RE62141/RE62149). E2 levels were in the ranges suggest by IBL (Table 1). The use of salivary E2 can be justified by the fact that salivary E2 strongly correlates with free serum E2 (Dielen et al., 2019), which is the portion available for estrogenic effects (Wu et al., 1976; Zhang and Ho, 2011).

Procedure and Statistical Analyses

We first compared *ESR1* methylation levels of the three targeted DNA regions among menopausal groups, using analysis of variance (ANOVA). If Levene's test was significant, the Welch statistics were used, followed by the Games–Howell test for post-hoc comparisons. ANOVA was conducted using SPSS (IBM statistic, version 24.0, Armonk, NY, USA: IBM Corp.). Next, we assessed the predictive effect of E2 levels and age on *ESR1* methylation using the robust regression approach. This method allowed to put less weight on more extreme values, which did not present compelling reasons justifying their exclusion (i.e., low sequencing coverage). Menopausal status was also included as a covariate in the regression models. In a second step, we added the interaction term between E2 and menopausal status for assessing possible differential associations between *ESR1* methylation and E2 among menopausal groups. The comparison of the different associations was performed using contrast analysis. Robust regression analyses were conducted using the R-Package 'robustbase' (Maechler et al., 2018) in R, version 3.5.0 (R Development Core Team, 2008). All methylation analyses were performed using the mean methylation of the 27, 12, and 9 CpGs in promoter A, promoter B, and CpGI shore, respectively, and methylation levels at individual CpGs. For most analyses using individual CpGs, only significant results were reported. One significant result, with an effect size < 0.04 was considered meaningless (Sullivan et al., 2012). All statistical tests were two-tailed and the significance level was set at $p < 0.05$.

3.1.3 Results

Description of Demographic and Biological Measures

The final study population comprised 130 women aged 40–73 years. All women were Caucasian, most were originally from Switzerland (89%) and the remaining part (11%) from

the neighboring German-speaking countries, Germany, Austria, and Liechtenstein. Of the total sample, 39.2% (n = 51) were premenopausal, 12.3% (n = 16) perimenopausal, and 48.5% (n = 63) postmenopausal. The majority of the women were married or living together with their partner (68.5%) and had a college/university degree or vocational education (79.2%). Table 2 presents the descriptive statistics of all variables used in this study, in the overall sample and according to menopausal groups.

Table 2: Demographic and biological measures.

	All	PRE	PERI	POST
N	130	51	16	63
E2 (pmol/L) (mean/SD)	5.6/4.5	8.2/4.7	5.7/4.5	3.7/3.2
Age (y) (mean/median/range)	53.2/40-73	45.1/45/40-57	51.4/51/47-56	60.2/59/50-73
ESR1 CpGI shore methylation (%) (mean/SD)	76.7/12	79.3/8.4	78.1/9.2	74.4/14.7
ESR1 promoter B methylation (%) (mean/SD)	3.5/3.14	3.2/2	3.9/3.3	3.7/3.8
ESR1 promoter A methylation (%) (mean/SD)	3.9/3.3	3.7/2.5	5/4	3.7/3.7

Note: levels of E2 in pre- and perimenopausal women are measured during the early follicular phase. Abbreviations: PRE= premenopausal; PERI= perimenopausal; POST= postmenopausal; E2= estradiol; *ESR1*= estrogen receptor 1 gene, CpGI= CpG island, SD= standard deviation.

ESR1 Promoter Methylation and Menopausal Status

No statistically significant differences between menopausal groups were detected when analyzing mean methylation of CpG island (CpGI) shore (F (2, 48.032) = 2.08, p = 0.137), mean methylation of promoter B (F (2, 39.126) = 0.64, p = 0.535), and mean methylation of promoter A (F (2, 127) = 1.02, p = 0.362). However, the examination of individual CpGs revealed that methylation at CpG9 of CpGI shore differed significantly between the groups (F (2, 43.953) = 5.08, p = 0.010), Figure 2A. Post-hoc analyses indicated significantly lower CpG9 methylation in postmenopausal women (71.1% ± 3.35 SE) compared to premenopausal women (83% ± 2.09 SE, p < 0.01). In addition, a significant difference in methylation was detected between groups at CpG7 of promoter B (F (2, 61.065) = 4.58, p = 0.014). Methylation at CpG7 was higher in postmenopausal women (3% ± 0.68 SE) than in premenopausal women (1.33% ± 0.25 SE, p = 0.060) and perimenopausal women (0.77% ± 0.3 SE, p = 0.010). The examination of methylation at individual CpGs of promoter A with respect to menopausal groups did not reveal any statistically significant differences.

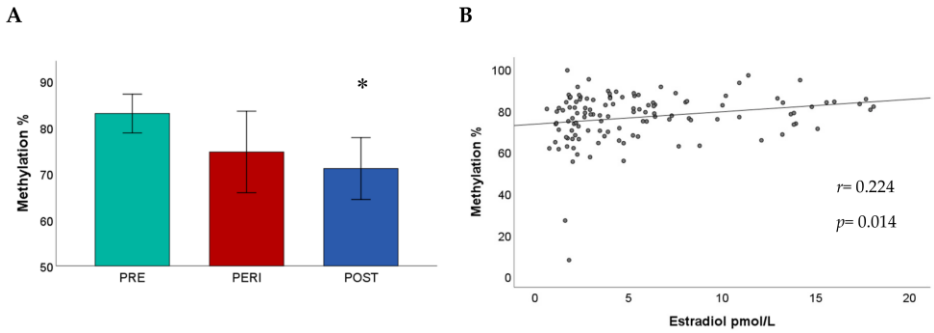


Figure 2: (A) Cytosine-phosphate-guanine island (CpGI) shore methylation at CpG9 in menopausal groups. Methylation levels are significantly lower in postmenopausal women compared to premenopausal women. (B) The mean methylation of CpGI shore is significantly positively associated with E2 levels (r = 0.224, p = 0.014). Robust regression was used to put less weight on extreme values. * p < 0.05. Abbreviations: PRE = premenopausal; PERI = perimenopausal; POST = postmenopausal.

ESR1 Promoter Methylation, Estradiol Levels, and Age

The results indicated that E2 levels were significantly predictive of CpGI shore mean methylation (β = 0.37, t (118) = 2.14, p = 0.034 Figure 2B), while age did not make any significant contribution (β = −0.04, t (118) = −0.22, p = 0.830). The examination of individual CpGs indicated that E2 levels were predictive of methylation at CpG3 (β = 0.54, t (118) = 2.39, p = 0.018) and CpG9 (β = 0.62, t (118) = 2.38, p = 0.019). Table 3 presents the effects of E2 levels and age on methylation at individual CpGs.

Table 3: Effects of estradiol (E2) levels and age on the *ESR1* CpGI shore methylation at individual CpGs.

	E2	*β*	*p*	*Age*	*β*	*p*
CpG 1		0.64	0.097		-0.25	0.599
CpG 2		0.12	0.733		-0.42	0.154
CpG 3		0.54	0.018		0.09	0.618
CpG 4		-0.01	0.978		-0.00	0.989
CpG 5		0.42	0.051		0.09	0.661
CpG 6		0.13	0.587		0.14	0.293
CpG 7		0.28	0.100		0.04	0.831
CpG 8		-0.06	0.797		-0.28	0.422
CpG 9		0.65	0.019		-0.33	0.419

Note: methylation at CpG 3 and CpG 9 present significant associations with E2 levels (in bold), while the same association at CpG 1 and CpG 5 presents a trend toward significance. Abbreviations: CpG = cytosine-guanine dinucleotide, E2 = estradiol.

Mean methylation of promoter B was not significantly predicted by E2 levels (β = −0.03, t (121) = −1.05, p = 0.294) or by age (β = −0.04, t (121) = −1.58, p = 0.117). However, methylation at CpG 12 was significantly positively predicted by age (β = 0.05, t (121) = 2.65, p < 0.01).
Mean methylation of promoter A was not significantly predicted either by E2 levels (β = −0.04, t (123) = −1.00, p = 0.318) or by age (β = 0.03, t (123) = 0.72, p = 0.471). Likewise, no significant difference in methylation was observed when examining individual CpGs.

CpGI Shore Methylation and Estradiol Levels Among Menopausal Groups

The regression model predicting CpGI shore mean methylation and including the interaction term between E2 and CpGI shore methylation was found to fit the data better than the model without the interaction term (F (2, 115) = 11.22, p = 0.004). A significant positive association between methylation and E2 was found in premenopausal (β = 0.56, t (113) = 2.71, p = 0.008) and in postmenopausal women (β = 0.55, t(113) = 2.04, p = 0.044), and no difference emerged between these two groups (p = 0.987). By contrast, a significant negative association between methylation and E2 was detected in perimenopausal women (β = −0.64, t (113) = −2.26, p = 0.026). The examination of individual CpGs indicated that the differential association was stronger at CpG 1 (p < 0.001).

3.1.4 Discussion

In this study, we explored DNA methylation of key regulatory regions of *ESR1* in association with menopausal status, age, and E2 levels in healthy middle-aged and older women. Methylation levels were low at promoters A and B, while intermediate methylation levels were found at CpGI shore. Postmenopause and lower E2 levels were associated with lower methylation of CpGI shore, and the effect of E2 levels was significant also after adjusting for menopausal status and age. This association was stronger at CpG 3 (Illumina probe: cg07746998), CpG 9 (Illumina probe: cg17264271), and CpG 1 (not included among Illumina probes). CpGI shore methylation was positively associated with E2 levels in pre- and postmenopausal women, while this association was negative in perimenopausal women. Postmenopause was associated with increased methylation of promoter B at CpG 7 (Illumina probe: cg22839866), while age was positively associated with methylation of promoter B at CpG 12 (Illumina probe: cg13612689) after adjusting for E2 levels and menopausal status. Concerning the methylation of promoter A, we did not observe an association with menopausal status, E2 levels, or age.

Our findings on *ESR1* methylation associated with E2 levels and age present similarities with previous research. First, decreased methylation of CpGI shore at the same CpGs region targeted in the present study was associated with increased age and ovariectomy in the hippocampus (region CA1) of female rats, as well as with E2 deprivation in human breast cancer cells non-resistant to hormone therapy (Ianov et al.; Tsuboi et al., 2017). Our results add to these findings and suggest similar associations in peripheral blood cells of healthy women. Second, in the study by Tsuboi et al. (2017), decreased methylation of proximal promoters was not found to be associated with E2 deprivation. Similarly, in our study, decreased methylation of promoter A and promoter B was not associated with menopausal status or E2 levels. On the contrary, consistent with studies showing that methylation at proximal promoters increases with aging (Issa et al., 1994; Post et al., 1999), increased methylation of CpG 7 and CpG 12 in promoter B was associated with postmenopause and increased age, respectively. Thus, contrary to CpGI shore, *ESR1* promoters A and B may not lose methylation in conditions of lower E2 levels, such as increased age and postmenopause. As increased methylation of *ESR1* promoter region (including CpGI shore) has been associated with decreased levels of ERα and increased incidence of age-related diseases (Gaudet et al., 2009; Issa et al., 1994; Kirn et al., 2018; Post et al., 1999; Sheng et al., 2017), hypomethylation of CpGI shore in older age may represent a health-promoting mechanism.

The CpGI shore of promoter C assessed in this study has been described as an enhancer (enhancer ID GH06J151804) of targeted promoters, including promoter A (Fishilevich et al., 2017; Tsuboi et al., 2017). Enhancers are regulatory DNA regions that increase gene transcription by influencing the activity of their target promoters (Pennacchio et al., 2013). DNA methylation has been shown to regulate the activity of enhancers, with methylation loss contributing to their activation (Bell et al., 2020; Lin et al., 2020; Stadler et al., 2011). Methylation dynamics at enhancers, marked by intermediate levels of methylation, has also been suggested as a mechanism by which the cell responds to environmental influences, including endogenous changes related to aging (Avrahami et al., 2015; Magnusson et al., 2015). We found intermediate levels of methylation in the CpGI shore, suggesting enhancer activity of this sequence in blood. Moreover, decreased methylation at enhancers has been

found to correlate with better cell function during aging (Avrahami et al., 2015). These findings may further support the idea that decreased CpGI shore methylation is associated with health during aging.

DNA methylation changes that reflect a programmed process are perhaps selected through evolution (Field et al., 2018). There is increasing evidence indicating that the hypomethylation of enhancers may be an example of these programmed DNA methylation changes (Field et al., 2018). For instance, hypomethylation of enhancers has been implicated as a component of the mouse clock (Field et al., 2018), and, as mentioned above, has been shown to preserve cell-functions in mouse pancreatic β-cells during aging (Pinzone et al., 2004). Hormonal changes related to menopause have been described as potential determinants of the epigenetic clock (Jylhävä et al., 2017). In this study, we described an association between E2 levels and ERα enhancer methylation that may promote healthy aging. In an evolutionary context, this hormone-DNA methylation association would have positive effects on fitness-related traits earlier in life, as the strength of natural selection decreases with age (Fabian et al., 2011).

In our sample, the positive association between CpGI shore methylation and E2 levels observed in pre- and postmenopausal women was not verified in perimenopausal women. This observation may be traced to the previously suggested idea that dysregulation of the estrogen signaling and epigenetic alterations of *ESR1* occur during perimenopause (Brinton et al., 2015).

DNA methylation of the CpGI shore may influence mRNA expression by regulating the binding of transcription factors sensitive to DNA methylation. For instance, binding sites for transcription factors of the ETS family are identified in highly conserved regions of the CpGI shore, which include CpG 1, CpG 2, CpG 4, CpG 5, CpG 7–9 (Kreft et al., 2017; Tsuboi et al., 2017). Furthermore, a STAT5b binding site is found in a region including CpG 3 (Champagne et al., 2006). Transcription factors of the ETS family and STAT5b are repressed from binding by methylation within their binding sites in the CpGI shore, leading to decreased *ESR1* expression (Champagne et al., 2006; Héberlé and Bardet, 2019; Tsuboi et al., 2017). This supports the assumption that DNA methylation differences of CpGI shore found in the present study play a role in regulating CpGI shore transcription.

Finally, our results indicate that E2 was the unique predictor of CpGI shore methylation when controlling for age and menopausal status. E2, through ER, has been shown to exert epigenetic influence on various genes in different tissues, including the blood (Campesi et al., 2012; Zhang and Ho, 2011). E2 levels may also contribute to regulate *ESR1* CpGI shore methylation. However, the mechanism underlying the potential regulation of CpGI shore methylation by E2 has not yet been elucidated. As discussed by Ianov et al. (2017), the complex E2-ERα may enhance transcription of repressors interacting with methyltransferases, which in turn would add methyl groups at CpGs of CpGI shore. Thus, a feedback mechanism involving ERα, transcription repressors, and methyltransferases may underlie the association between CpGI shore methylation and E2 levels.

Strengths and Limitations

This is the first study to explore associations between *ESR1* promoter methylation and E2 levels in the context of women healthy aging. In addition, during the participants' recruitment process, strict inclusion and exclusion health criteria were applied. Therefore, the results could not have been biased by major illnesses.

Although there is evidence indicating that increased CpGI shore methylation is associated with decreased *ESR1* expression in various tissues (Champagne et al.,2006; Gaudet et al., 2009; Ianov et al. 2017; Tsuboi et al., 2017), limitations of this study include the lack of assessment of *ESR1* expression. Furthermore, we assessed DNA methylation only in peripheral blood. This prevents the generalization of results, as DNA methylation may be tissue-specific (Hannon et al., 2015). However, the blood DNA methylation as a proxy of physiological processes in other tissues has been previously demonstrated (Smith et al., 2014). In addition, an epigenome-wide analysis showed that DNA methylation in blood was predictive of all-cause mortality in a sample of 9949 older adults aged 50–75 years (Zhang et al., 2017). Moreover, the *ESR1* promoter methylation in blood has proved useful in the diagnosis of lung and breast cancers (Khakpour et al., 2015; Martínez-Galán et al., 2014; Suga et al. 2008). Furthermore, it should be noted that methylation at *ESR1* CpGI shore in blood has been found to correlate with the *ESR1* CpGI shore methylation in the brain, especially in the superior temporal gyrus (Supplementary Figure S1) (Hannon et al., 2015). Nevertheless, for future studies it would be important to explore the association between CpGI shore methylation, E2 levels, and *ESR1* expression in different cell types. Another limitation is the lack of a longitudinal study design. Indeed, longitudinal data would allow the identification of changes of CpGI shore methylation following the individual variations in E2 levels, age, and menopausal status. Moreover, the sample of perimenopausal women was small (n = 16) compared to pre- (n = 51) and postmenopausal (n = 63) women. Therefore, findings regarding the perimenopausal group must be interpreted with caution. At last, in this study, we did not assess data linked to women's nutritional status, such as B12 and red cell folate, which have been shown to regulate DNA methylation (Mahajan et al., 2019). In conclusion, ERα plays an important role in maintaining health during aging. This report indicates that decreased methylation of *ESR1* CpGI shore may be associated with conditions of lower E2 in older healthy women. This might have important clinical implications in the field of women healthy aging. Future research on this topic may consider gene expression analysis, longitudinal cohorts, and cell-specificity.

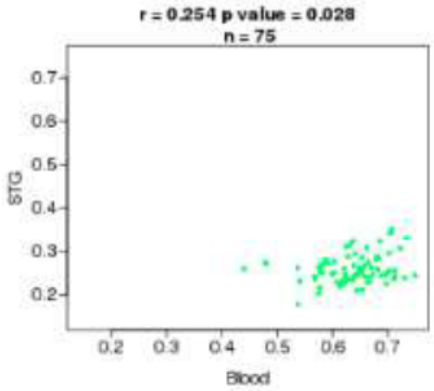
3.1.5 Acknowledgments

Data produced and analyzed in this paper were generated in collaboration with the Genetic Diversity Centre (GDC), ETH Zurich. We also wish to thank Sarah Mannion (proofreading) and Dries Deeber (statistical consulting). This research was funded by the University Research Priority Program (URPP), Dynamics of Healthy Aging, University of Zurich, Switzerland.

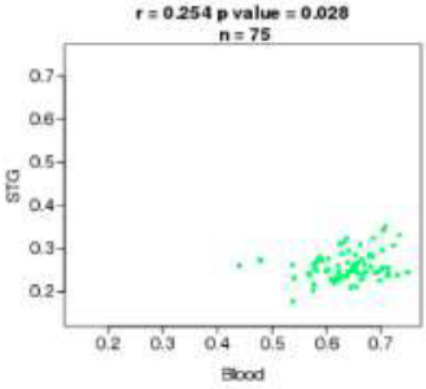
3.1.6 Supplementary Information

Supplementary Figure S1. Correlation of *ESR1* CpGI shore methylation between blood and the superior temporal gyrus (STG) (Hannon et al., 2015). The CpGs presented in the figure are those included as probes in the Illumina system (Illumina 450K probe).

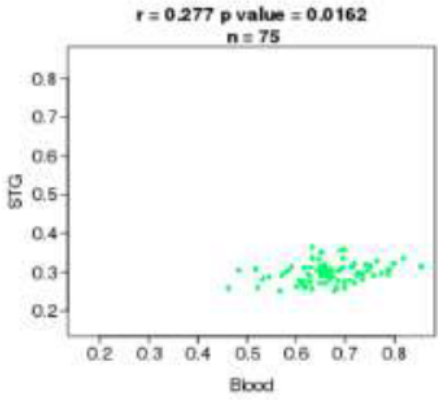
CpG 2 (cg20893956)



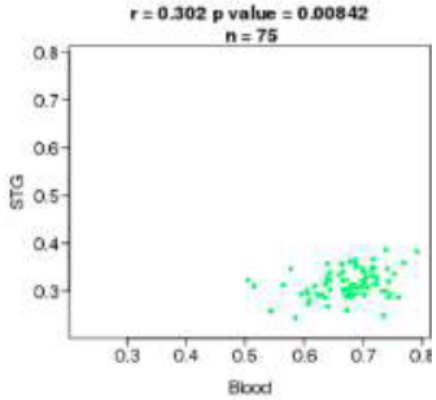
CpG 3 (cg24764793)



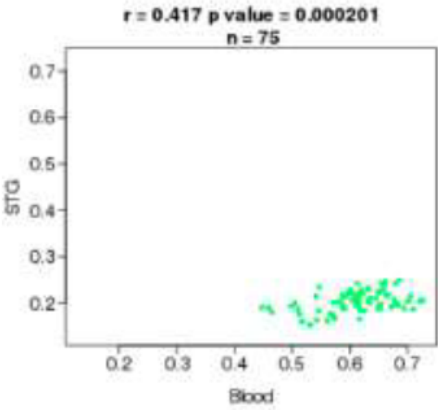
CpG 4 (cg07746998)



CpG 8 (cg21157690)



CpG9 (cg17264271)



3.2 Study II: Sleep and Methylation of Estrogen Receptor Genes, *ESR1* and *GPER*, in Healthy Middle-Aged and Older Women: Findings from the Women 40+ Healthy Aging Study

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Purpose: Sleep problems in middle-aged and older women are very common and have been associated with menopause-related changes in estrogen levels. However, not all women experience sleep problems as they enter perimenopause, and epigenetic mechanisms might contribute to the differences in sleep quality within this population. In this study, we hypothesized that increased methylation of two estrogen receptor (ER) genes (*ESR1* and *GPER*) would be associated with increased sleep problems in healthy pre-, peri-, and postmenopausal women, either directly or indirectly through the experience of vasomotor symptoms (VMS). **Materials and Methods:** In 130 healthy women aged 40– 73 years, we assessed DNA methylation from dried blood spots (DBS). Women rated their sleep quality using the Pittsburgh Sleep Quality Index (PSQI), and VMS using the Menopause Rating Scale (MRS). **Results:** Higher percentage methylation of *ESR1* was associated with increased sleep problems, mediated by VMS, even after controlling for age, menopausal status, body mass index, estradiol levels, depressive symptoms, and caffeine consumption. There was no significant association between *GPER* methylation and either sleep problems or VMS. **Conclusion:** The study findings support an association between increased *ESR1* methylation and sleep problems through increased VMS among healthy women aged 40– 73 years.

Keywords: sleep problems, VMS, *ESR1*, *GPER*, DNA methylation, healthy middle-aged and older women

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3.2.1 Introduction

Sleep is an important determinant of quality of life during aging, even among healthy individuals (Driscoll et al., 2008; Dew et al., 2003). Sleep problems in the form of insomnia, difficulty falling asleep, lower sleep efficiency, or frequent and early awakening become increasingly common with advancing age, and affect as many as 40–56% of middle-aged and older women (Baker et al., 2018). Menopausal status (MS) is strongly associated with sleep problems, with peri- and postmenopausal women reporting more sleep problems than premenopausal women (Jehan et al., 2015). Changes in levels of ovarian hormones such as estrogens are important contributors to the occurrence and severity of sleep problems as women enter perimenopause (Carrier et al., 2017). Indeed, besides their role in women's reproductive functions, estrogens exert a vast range of biological effects. In the central nervous system, estrogens contribute to the control of circadian rhythms and body temperature (Carrier et al., 2017; Vrtačnik et al., 2014), and their declining and fluctuating levels have been repeatedly associated with sleep problems and vasomotor symptoms (VMS) (Al-Safi and Santoro, 2014; Carrier et al., 2017; Hollander et al., 2001; Landis and Moe, 2004; Santen et al., 2010; Vrtačnik et al., 2014). VMS, such as hot flashes and night sweats, are primarily thermoregulatory phenomena and are the main cause of sleep problems (Baker et al., 2018; Pengo et al., 2018). Although VMS are a hallmark of perimenopause, they can persist for several years after the last menstrual period. Therefore, they may continue to affect sleep even in advanced age (Freeman et al., 2014). However, while all aging women experience major hormonal changes, not all women experience increased sleep problems (Thurston and Joffe, 2011). This suggests that genetic and environmental factors may play a role in the exacerbation of sleep problems among middle-aged and older women (Barclay and Gregory, 2013; Davis et al., 2015; Schneiderman et al., 2005; Vézina-Im et al., 2017; Ziv-Gal et al., 2010).

Epigenetic modifications are increasingly recognized as potential mechanisms underlying the etiology and phenotypic variation of multiple diseases (Moosavi and Ardekani, 2016). It has also been suggested that epigenetic modifications may constitute underlying mechanisms of the gene-environment interplay affecting sleep and menopause-related symptoms (Barclay and Gregory, 2013; Brinton et al., 2015; Kim et al., 2012). DNA methylation is a key epigenetic mechanism, which regulates gene expression through DNA modifications at cytosines in cytosine-guanine dinucleotides (CpGs) (Kumar et al., 2018). Increased DNA methylation of regulatory regions of estrogen receptor (ER) genes has been shown to decrease levels of ERs (Champagne et al., 2006; Tang et al., 2019; Vrtačnik et al., 2014). ERs mediate the effects of estrogens in the body, including the estrogenic contributions to the control of circadian rhythms and body temperature (Frank et al., 2014; Kelly and Rønnekleiv, 2014; Liu and Shi, 2015). While the ERα and the ERβ mainly mediate the genomic effects of estrogens, the more recently discovered G protein-coupled estrogen receptor 1 (GPER) mediates rapid non-genomic estrogen effects (Prossnitz and Barton, 2011). Several lines of evidence from animal studies indicate that decreased levels of the three ERs may contribute to increase sleep problems and VMS (Hatcher et al., 2019; Kelly and Rønnekleiv, 2014; Qiu et al., 2003).

To the best of our knowledge, no study has yet investigated the associations between ER gene methylation and sleep problems in middle-aged and older women. DNA methylation levels of specific regions of ER genes may contribute to alter sleep quality among women as they reach midlife. Therefore, in this study, we hypothesized that increased levels of ER gene methylation

would be associated with increased sleep problems in healthy pre-, peri-, and postmenopausal women, either directly, or indirectly through VMS. We focused on the ERα gene (*ESR1*) and GPER gene (*GPER*) based on knowledge of the involvement of both genomic and non-genomic estrogen effects in sleep problems and VMS, and given the presumed greater role of ERα compared to ERβ in controlling circadian rhythms and body temperature in women (Hatcher et al., 2019; Gouw et al., 2017).

3.2.2 Materials and Methods

Inclusion and Exclusion Criteria

Women who took part in this study were recruited in the context of a larger investigation, the "Women 40+ Healthy Aging Study" (Fiacco et al., 2019; Fiacco et al., 2020; Gardini et al., 2020; Mernone et al., 2019). Women were recruited using flyers, social media (e.g., Facebook), and journal articles. The prospective participants completed an online self-screening to verify their eligibility for the study. Women were included in the study if they reported being free of any acute or chronic somatic disease or mental disorder, and if they had not received any psychotherapeutic or psychopharmacological treatment during the last 6 months. In addition, women had to report good, very good, or excellent subjective health status. Further exclusion criteria were a pregnancy in the last 6 months, menopause due to surgical removal of the ovaries or the uterus, premature menopause, current use of oral contraceptives or hormone therapy. After completion of the online self-screening, eligible women were reached telephonically by a member of the study team. The telephone call aimed, first, to ensure that only women suitable for the study were included, and second, to answer open questions about the study procedure. Participants were invited to a laboratory session during which saliva and blood samples were collected. All sessions started at 7:45 a.m. and followed a standardized protocol.

Participants

In total, 130 healthy Caucasian women aged 40–73 years took part in the study. The women lived in Switzerland, and the majority were Swiss or from a German-speaking neighboring country. Most of the women were married or living together with their partner (68.5%), and had vocational education or a college/university degree (79.2%). Participants were divided into three subgroups with respect to their MS according to the Stages of Reproductive Aging Workshop +10 (STRAW) criteria (Harlow et al., 2012): 1) premenopausal (39.2%, n= 51), if the menstrual cycle was regular, 2) perimenopausal (12.3%, n= 16), if the cycle length was variable, with a variability among cycles of at least 7 days, or if the interval between cycles was > 60 days, and 3) postmenopausal (48.5%, n= 63), if no bleeding had occurred in at least the last 12 months. Women using hormone therapies for reducing menopausal symptoms were excluded during the online self-screening. After enrolment, women were screened for the use of other medications. Five women reported taking botanical therapies such as Cimifemin® (2), Cimifemin® forte (1), Premens (1), and a plant-based preparation (no pharmaceutical name provided; 1). In addition, one woman reported taking Eltroxin (hypothyroidism) and one woman reported taking Tamoxifen (breast cancer prevention). The results were not affected by the exclusion of these participants from the statistical analyses, and the sample of this study includes participants reporting the use of the above-mentioned

medications. After receiving written and oral information about the study, all participants signed written informed consent forms. The study (BASEC Nr 2016–01591) was evaluated by the cantonal ethics committee of the Canton of Zürich (KEK Zürich, Zürich, Switzerland). Approval from the cantonal ethics committee was not required because the assessment of relationships between biological and physiological parameters in healthy aging individuals was not considered to fall within the scope of the "Humanforschungsgesetz" (HFG) (Art. 2 HFG and Art. 3 HFG).

Sleep Problems

Sleep problems were assessed using the German version of the Pittsburgh Sleep Quality Index (PSQI) (Buysse et al., 1989). The scale assesses sleep problems over a one-month time interval and comprises 19 items combined into seven component scores: perceived sleep quality ("how would you rate your sleep quality overall"?), sleep latency (eg: "cannot get to sleep within 30 minutes"), sleep duration ("how many hours of actual sleep do you get at night"?), sleep efficiency (number of hours slept/number of hours spent in bed), sleep disturbances (eg: "wake up in the middle of the night or early morning", "had bad dreams"), use of sleep medication (eg: "how often have you taken medicines to help you sleep"?), and daytime dysfunction (eg: "how much of a problem has it been for you to keep up enough enthusiasm to get things done?"). Each component is weighted equally from 0 to 3, and the seven component scores are summed up to obtain a total score ranging from 0 to 21. A score above 5 is considered a marker of sleep problems. The single total score is frequently used in the literature, and acceptable reliability and validity in different populations have been reported (Otte et al., 2015). However, the model including only perceived sleep quality, sleep efficiency and daytime dysfunction (3-factor model) seems to better reflect good versus poor sleep quality in healthy peri- and early postmenopausal women with hot flashes, and in older adults (Otte et al., 2015; Cole et al., 2006). Therefore, in the statistical analyses of the present study, we used both the total score and the 3-factor model of the PSQI. Descriptive statistics of sleep problems in the overall sample and based on menopausal groups are reported in Table 1.

Table 1: Descriptive Statistics

	All	PRE	PERI	POST
N	130	51	16	63
Sleep problems (mean/SD)	4.8/2.5	4.2/2.4	5.8/2.6	5/2.5
VMS (mean/SD)	1.8/1	1.3/0.5	2.4/1.1	2/1.2
ESR1 methylation (%; mean/SD)	76.7/12	79/8.4	78.1/7.9	74.5/14.7
GPER methylation (%; mean/DS)	15.3/7	14.8/1.8	19.2/3.8	14.8/1.8
Age (mean/range)	53.2/40-73	45.1/40-57	51.4/47-56	60.2/50-73
BMI (kg/m^2; mean/SD)	23/3.6	22.8/3.9	21.5/3.4	23.7/3.3
E2 (nmol/L; mean/SD)	5.6/4.5	8.2/4.7	5.7/4.5	3.7/3.2
Depressive symptoms (mean/SD)	8.3/5.9	7.4/5.5	10.1/7.3	8.6/5.7
Caffeine (cups/day; mean/SD)	2.5/2	2.2/0.2	2.1/0.4	2.7/0.1

Note: The percentages of *ESR1* and *GPER* methylation correspond to the mean of methylation percentages of the 9 CpGs and 22 CpGs assessed in the *ESR1* and *GPER* sequences, respectively.
Abbreviations: PRE= premenopausal, PERI= perimenopausal, POST= postmenopausal, VMS= vasomotor symptoms, *ESR1*= estrogen receptor 1 gene, *GPER*= G protein-coupled estrogen receptor gene, BMI= body mass index, E2= estradiol, SD= standard deviation.

Vasomotor Symptoms

VMS were measured using one item ("hot flashes/sweating") of the somato-vegetative subscale of the German version of the Menopause Rating Scale (MRS) (Heinemann et al., 2003). VMS are rated on a 5-point Likert scale, ranging from "0" (no symptoms) to "4" (very severe symptoms). The prevalence of VMS was assessed as follows: 0 (no symptoms), 1–4 (symptoms). Descriptive statistics of VMS for all women and separately for menopausal groups are reported in Table 1.

Blood Sampling

Blood samples were collected at 8:00 am during the laboratory session at the University of Zurich under standardized conditions. For pre- and perimenopausal women, sampling was conducted in the early follicular phase. Blood was collected from fingertips and spotted onto S&S 903 Whatman® paper cards (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), following the manufacturer's instructions. Blood spots were dried at room temperature for approximately 3 h. Dried blood spots (DBS) were stored at −20°C until biochemical analysis.

DNA Methylation

Genomic DNA was extracted from DBS, using the QIAamp DNA Investigator Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions, and eluted in a final volume of 30 µL of RNase-free water. We obtained a total DNA yield ranging from 41 to 168 ng, as measured with Qubit (Thermo Fischer Scientific, Waltham, MA, USA). The DBS technology was reported to provide highly reliable results in the context of methylation studies (Aberg et al., 2013). Genomic DNA (41–168 ng) was bisulfite-converted using the EZ 96-DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA) and eluted in 20 ul of water. A first amplification was performed on the bisulfite-converted DNA using the Kapa HIFI Uracil+ master mix and primers that were designed manually or using the MethPrimer software.43 Primers (Table 2) included the universal primer sequences CS1/CS2 at the 5' ends of the forward and reverse primers, and were designed in order to target a specific sequence of the *ESR1* shore of promoter C (hg 38; chr6:151805523–151805822, Figure 1A) and the *GPER* promoter (hg 38; chr7:1087059 −1087533, Figure 1B). DNA methylation in these regions was previously associated with altered gene expression and function (Tsuboi et al., 2017; Weissenborn et al., 2017). The PCR was performed with the following parameters: 95°C for 3 min [98°C for 20 sec – annealing temperature for 15 sec – 72°C for 15 sec] x 40 cycles, 72°C for 40 sec. In order to certify that primers were specifically amplifying bisulfite-converted DNA, genomic DNA was included in the PCR, and complete conversion was confirmed by including wild primers. Next, the PCR amplicons were purified using the E-gels 2% size selection (Thermo Fisher Scientific, Waltham, MA, USA). Purified amplicons were indexed with a single barcode (Fluidigm, San Francisco, California, USA) using a second PCR of 10 cycles (annealing temperature, 60°C). Indexed amplicons were pooled and again purified. The purified library was then quantified using the Agilent 2200 Tape Station instrument, with HS DNA 1000 reagents (Agilent Scientific Instruments, Santa Clara, CA, USA), and finally diluted to the concentration of 2nM. The final library was denatured in NaOH, mixed with 12% PhiX, diluted to 12 pM in HT1 buffer, and finally sequenced on an Illumina MiSeq sequencer using the v3 kit (300 base pairs paired-end).

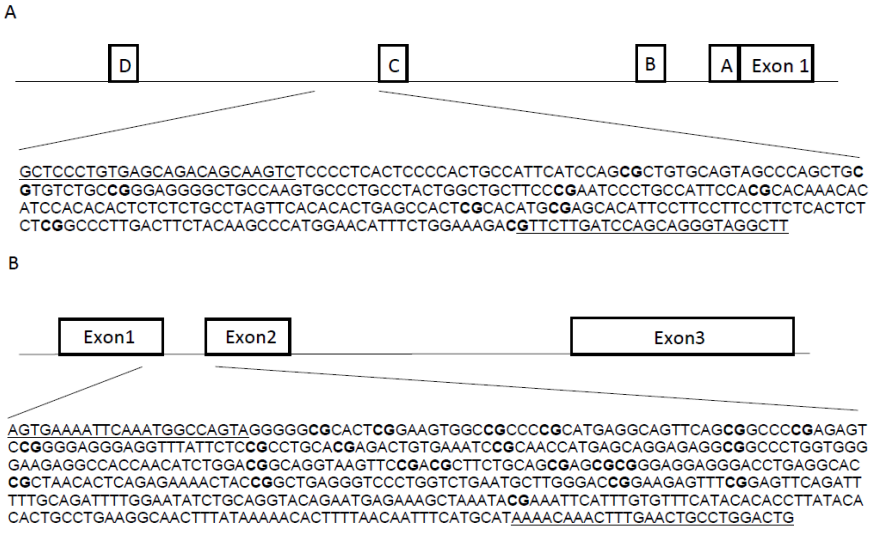

Figure 1: Schematic figures of *ESR1* (A) and *GPER* (B) promoter regions. (A) The DNA sequence of *ESR1*, chr6: 151805523–151805822, is located in a CpG island shore of promoter C, and it has been described as an enhancer (ID GH06J151804) of promoter A (Tsuboi et al., 2017). This region has been reported in humans and rats by Champagne et al, 2006; Gardini et al, 2020; Ianov et al, 2017; and Tsuboi et al, 2017. (B) The DNA sequence of *GPER*, chr7:1087059–1087533 is located in a CpG island across exons 1 and 2. This region has been assessed in Weissenborn et al, 2017. White boxes represent exons or promoter regions. Underlined sequences correspond to the primers position. Bold "CG" correspond to the Interrogated CpGs.

Table 2: PCR primers used for amplification of DNA sequences in the *ESR1* and *GPER*

Gene	Forward primer	Reverse primer	GRCh38	Ta (°C)
ESR1	ACACTGACGACATGGTTCTACA NNN GTTTTTTGTGAGTAGATAGTAAGTT	TACGGTAGCAGAGACTTGGTCT NNN AAACCTACCCTACTAAATCAAAAAC	chr6:151,805,523-151,805,822	58
GPER	ACACTGACGACATGGTTCTACA NNNAGTGAAAATTTAAATGGTTAGTA	TACGGTAGCAGAGACTTGGTCT NNNACAATCCAAACAATTCAAAATTTATTT	chr7:1,087,059-1,087,533	60

Note: Universal primer CS1= ACACTGACGACATGGTTCTACA, universal primer CS2= TACGGTAGCAGAGACTTGGTCT.
Abbreviations: PCR= polymerase chain reaction; *ESR1*= estrogen receptor alpha gene; *GPER*= G protein-coupled estrogen receptor gene; GRCh38= Genome Reference Consortium Human Build 38 Organism; Ta= annealing temperature.

As described previously, adaptors and primers were trimmed from the reads using the default settings of Trimmomatic v0.35 (licensed under GPL V3 and available at http://www.usadellab.org/cms/index.php?page=trimmomatic), and low-quality reads defined by a Phred quality score <30 were also discarded (Bolger et al., 2014; Chen et al., 2017). Using Bismark program (v0.19.0), the remaining sequencing reads were aligned to the target regions and the number of unconverted and converted cytosine was extracted for each interrogated CpG. After summing up counts of unconverted and converted cytosine for each CpG, we removed all samples showing a coverage (=total count) lower than 100x. (Chen et al., 2017). For *ESR1*, six samples did not reach the 100x threshold and were therefore excluded. For the remaining samples, coverage ranged from 157 to 42'709 (Mean= 6'5601, SD= 5'968). The coverage of most samples for *GPER* was less than 100x, and only 34 samples could be retained in the analyses. For these 34 samples, coverage ranged from 350 to 39'942 (Mean= 10'647, SD= 9553). The methylation percentage for each CpG was calculated as the unconverted (cytosine) read count divided by the total read count (cytosine + thymine). The *ESR1* shore and *GPER* methylation data are openly available in "Dryad" at https://doi.org/10.5061/dryad.51c59zw51.

One CpG cluster was identified for *ESR1*, and therefore the mean of the nine assessed CpGs was used in the statistical analyses. For *GPER*, two main clusters were identified. However, the results were similar when using the two clusters or the mean of all 22 CpGs assessed for *GPER*. Therefore, only results obtained using the mean level across all 22 CpGs are presented in this study. Descriptive statistics of *ESR1* and *GPER* methylation levels are reported in Table 1 for the entire sample and the three menopausal stages, respectively.

Potential Covariates and Cofounders

In the statistical analyses we adjusted for age, MS, body mass index (BMI), levels of estradiol (E2), depressive symptoms, and caffeine consumption (Cappuccio et al., 2008; Field et al., 2018; Gold et al., 2006; Hollander et al., 2001; Kravitz et al., 2008; Landis and Moe, 2004; Levine et al., 2016; Matsuda, 2014; Miner and Kryger, 2017; Roehrs and Roth, 2008; Woods and Mitchell, 2010). Depressive symptoms were assessed using the German version of the Center for Epidemiological Studies Depression scale (CES-D), which is a commonly used self-report measure of depressed symptoms during the past one to 2 weeks.58 It consists of 20 items (eg, "I felt hopeful about the future", "I felt that people disliked me"), rated on a 4-point

Likert scale ranging from 0 (rarely or none of the time [less than 1 day]) to 3 (most or all of the time [five to 7 days]). The total score can range from 0 to 60, with higher scores indicating higher depressive symptoms. A cut-off score of 16 or above is often used as a marker of risk for clinical depression (Radloff, 1977). Levels of E2 concentrations were measured in saliva using an enzyme-linked immunosorbent assay (IBL international, Hamburg, Germany), following the manufacturer's instructions. Saliva samples were collected at 8:00 am into 2-mL SaliCaps (IBL International GmbH, Hamburg, Germany) immediately before the collection of blood samples. Intra- and inter-assay coefficients were below 13.3% and 14.8%, respectively, and the assay's analytical sensitivity was 1.1 pmol/L. Values greater than three standard deviations around the mean were considered as outliers (n= 4) and therefore removed from the analyses. Salivary E2 strongly correlates with free serum E2, which is the portion available for estrogenic effects (Dielen et al., 2019; Wu et al., 1976). BMI was calculated by dividing the weight in kilograms by the height in meters squared, and caffeine consumption was determined as the number of cups of coffee the women drink per day.

Procedure and Statistical Analyses

Descriptive statistics were calculated to assess the prevalence and severity of sleep problems and VMS among the three menopausal groups (pre-, peri and postmenopausal women). We used Chi-squared test in order to test the prevalence of sleep problems and VMS, and one-way analysis of variance (ANOVA) to test their severity. In the Chi-square test post-hoc analysis, p-values were estimated from unstandardized Z values, according to García-perez et al (2003) (García-pérez and Núñez-antón, 2003). A p-value below 0.05 was considered statistically significant.

In order to assess the effects of *ESR1*/*GPER* methylation on sleep problems, considering VMS as potential mediators, we conducted simple mediation analyses using the PROCESS macro (model 4) (Hayes, 2017) in SPSS. The mediation model allows the examination of the independent contribution of direct and indirect effects through three paths (a_i, b_i, c', see Figure 2): Path ai represents the association between the predictor (X) and the mediator (M_i), path b_i represents the association between M_i and the outcome variable (Y), and path c' represents the direct effect of X on Y, after adjusting for all other variables included in the model. Finally, the indirect effect of X on Y through M_i is defined as $a_i x b_i$. Direct and indirect effects were assessed using bootstrapping, with the b coefficient providing an index of the magnitude of the effect. Bootstrapping generated a confidence interval (CI). When the 95% asymmetric CI did not include the value 0, direct and indirect effects were considered significant. Bootstrapping has the advantages of not requiring normal distribution of data and of a low vulnerability to type 1 error (Hayes, 2017). In a second step, MS was dummy-coded, with the premenopausal group (compared to peri- and postmenopausal groups) and perimenopausal and postmenopausal groups (compared to the premenopausal group) as dummies, to investigate whether effects of the mediation analysis differed between premenopausal women and the group including peri- and postmenopausal women. All analyses were conducted using SPSS (IBM Statistics, version 24.0, Armonk, NY: IBM Corp.).

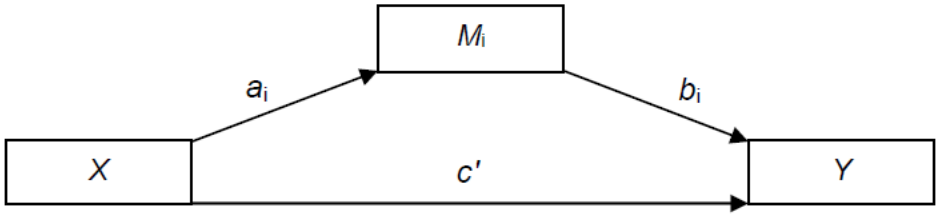

Figure 2: Path diagram (Macro Model 4) according to Hayes (2017)

3.2.3 Results

Levels of mean methylation across the investigated CpGs of *ESR1* and *GPER* for the three menopausal groups are provided in Table 1. In addition, all variables assessed in this study are provided in this table.

Descriptive Statistics of Sleep Problems Among Menopausal Groups

Almost one third of the sample (30.5%) experienced sleep problems (score of PSQI > 5) during the previous month. Sleep problems were reported by 24% (n= 12) of premenopausal women, 31.3% (n= 5) of perimenopausal women, and 35.5.% (n= 22) of postmenopausal women. The difference in the prevalence of sleep problems among the three menopausal groups was not statistically significant [$\chi 2$ (2, N= 128)= 1.73, p= 0.421]. However, there was a trend toward significance regarding the difference in the severity of sleep problems among the groups [F(2125)= 2.94, p= 0.056], with greater sleep problems in peri- (p= 0.028) and postmenopausal women (p= 0.087) compared to premenopausal women.

Descriptive Statistics of Vasomotor Symptoms Among Menopausal Groups

VMS were reported by 48.1% of all women. VMS were experienced by 27% (n= 14) of premenopausal women, 81.3% (n= 13) of perimenopausal women, and 56.5% (n= 35) of postmenopausal women. Among the three groups, there was a statistically significant difference in the prevalence of VMS [$\chi 2$ (2, N= 129)= 17.49, p< 0.001]. The number of premenopausal women experiencing VMS was significantly lower than the number of peri- and postmenopausal women experiencing VMS (p< 0.001). The difference in the severity of VMS among menopausal groups was also statistically significant [F (2126)= 10.38, p< 0.001], with peri- (p< 0.001) and postmenopausal women (p< 0.001) reporting greater VMS severity compared to premenopausal women. No significant difference in VMS severity was detected between peri- and postmenopausal women (p= 0.130).

ESR1 Methylation and Sleep Problems

Using the total score of the PSQI as outcome variable, the results indicated that *ESR1* methylation predicted sleep problems indirectly through VMS (b= 0.011, 95% CI [0.0013, 0.0299]), while the direct effect of *ESR1* methylation on sleep problems was not significant (b= 0.025, 95% CI [−.0100, 0.0597], Figure 3). The results of this mediation model are presented in Table 3. The results of the sensitivity analysis indicated that most of the

mediation effect came from peri- and postmenopausal women (b= 0.0127, 95% CI [0.0005, 0.0246]), compared to premenopausal women (b= 0.0045, 95% CI [−.0020, 0.0121]).

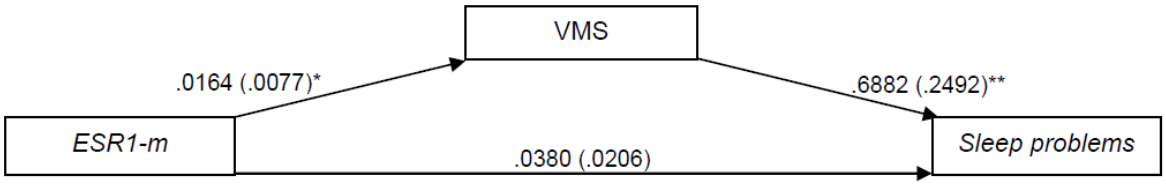

Figure 3: Path diagram of the mediation model (Macro Model 4),62 illustrating the associations between *ESR1* methylation (*X*) and sleep disturbances (*Y*) through VMS (M_i). Estradiol (E2), menopausal status (MS), depressive symptoms, body mass index (BMI), age and caffeine consumption were included in the model and controlled in each of the three paths (a_i, b_i, c'). Values outside parentheses= unstandardized *b* coefficient; values in parentheses= standard error; *= p-value < 0.05; **= p-value < 0.01.
Abbreviations: *ESR1*-m= estrogen receptor 1 methylation; VMS= vasomotor symptoms.

Table 3: Results of the mediation model with *ESR1* methylation as predictor, 95% bias-corrected confidence interval predicting sleep quality.

		b	se	*t*	*p*	LLCI	ULCI
Outcome variable: VMS							
	ESR1-m (a_i)	.0175	.0078	2.2550	.0261	.0021	.0329
CV							
	Age	-.0516	.0169	-3.0573	.0028	-.0850	-.0182
	MS	.7049	.1640	4.2969	.0000	.3798	1.0300
	BMI	.0194	.0149	1.2970	.1974	-.0102	.0490
	E2	-.0018	.0224	-.0790	.9372	-.0462	.0427
	Depressive symptoms	-.0151	.0155	-.9704	.3340	-.0458	.0157
	Caffeine	.0201	.0575	.3502	.7269	-.0938	.1340
Outcome variable: sleep problems (PSQI)							
	VMS (b_i)	.6301	.2112	2.9837	.0035	.2116	1.0487
CV							
	Age	.0586	.0389	1.5055	.1351	-.0186	.1358
	MS	-.3619	.3927	-.9215	.3588	-1.1401	.4164
	BMI	.0178	.0333	.5331	.5950	-.0483	.0838
	E2	.0147	.0497	.2962	.7676	-.0838	.1132
	Depressive symptoms	.2033	.0345	5.8904	.0000	.1349	.2717
	Caffeine	-.2224	.1274	-1.7456	.0837	-.4748	.0301
Direct/indirect effects on sleep problems (PSQI)							
	Direct effect (*c*')	.0249	.0176	1.4159	.1597	-.0100	.0597
	Indirect effect ($a_i x b_i$)	.0110	.0073			.0013	.0299

Note: a_i represents the association between *ESR1*-m and VMS; b_i represents the association between VMS and sleep quality; *c*' represents the direct effect of *ESR1*-m on *Y*, after adjusting for all other variables included in the model; $a_i x b_i$ represents the indirect effect of *ESR1*-m on sleep quality through VMS; *b* represents the unstandardized regression coefficient.
Abbreviations: se= standard error, BootLLCI= bootstrapping lower limit confidence interval; BootULCI= bootstrapping upper limit confidence interval; VMS= vasomotor symptoms, *ESR1*-m= estrogen receptor alpha gene methylation; CV= control variables; MS= menopausal status; BMI= body mass index; E2= estradiol.

Similar results were obtained when using the 3-factor model. The indirect effect of *ESR1* methylation on the global scores of perceived sleep quality, sleep efficiency and daytime dysfunction through VMS was statistically significant (b= 0.007, 95% CI [0.0014, 0.0187]), while the direct effect was not significant (b= 0.011, 95% CI [−.0103, 0.0324]).

GPER Methylation and Sleep Problems

Using the total score of the PSQI as outcome variable, the results indicated that *GPER* methylation was not predictive of sleep problems, either directly (b= 0.073, t(23)= 1.08, 95% CI [−.0664, 0.2124]), or indirectly through VMS (b= −.037, 95% CI [−.1083, 0.0416], Figure 4). The results of this mediation model are presented in Table 4.

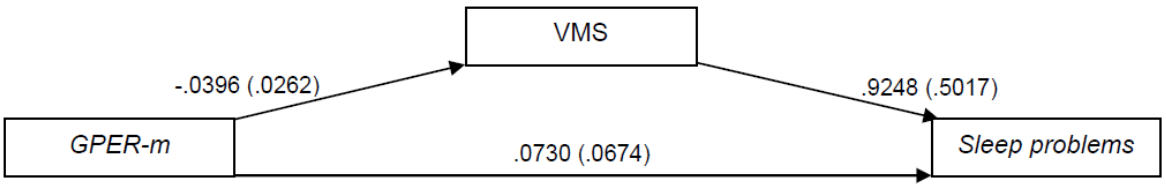

Figure 4: Path diagram of the mediation model (Macro Model 4),62 illustrating the associations between *GPER* methylation (*X*) and sleep disturbances (*Y*) through VMS (M_i). Estradiol (E2), menopausal status (MS), depressive symptoms, body mass index (BMI), age and caffeine consumption were included in the model and controlled in each of the three paths (a_i, b_i, c'). Values outside parentheses= unstandardized *b* coefficient; values in parentheses= standard error.
Abbreviations: *GPER*-m= G protein-coupled estrogen receptor methylation; VMS= vasomotor symptoms.

Table 4: Results of the mediation model with *GPER* methylation as predictor, 95% bias-corrected confidence interval predicting sleep quality.

		b	se	*t*	*p*	LLCI	ULCI
Outcome variable: VMS							
	GPER-m (a_i)	-.0396	.0262	-1.5117	.1437	-.0937	.0145
CV							
	Age	-.0749	.0353	-2.1208	.0445	-.1477	-.0020
	MS	.7074	.3276	2.1596	.0410	.0313	1.3836
	BMI	-.0210	.0245	-.8555	.4007	-.0716	.0296
	E2	-.0478	.0414	-1.1542	.2598	-.1332	.0376
	Depressive symptoms	-.0228	.0304	-.7485	.4614	-.0856	.0400
	Caffeine	.2073	.1481	1.3999	.1743	-.0983	.5129
Outcome variable: sleep problems (PSQI)							
	VMS (b_i)	.9248	.5017	1.8434	.0782	-.1131	1.9627
CV							
	Age	.1699	.0945	1.7971	.0855	-.0257	.3655
	MS	-1.2543	.8799	-1.4256	.1674	-3.0746	.5659
	BMI	-.0036	.0612	-.0594	.9531	-.1302	.1229
	E2	.0183	.1045	.1751	.8625	.1978	.2344
	Depressive symptoms	.2018	.0756	2.6686	.0137	.0454	.3583
	Caffeine	-.6193	.3785	-1.6360	.1155	-1.4023	.1638
Direct/indirect effects on sleep problems (PSQI)							
	Direct effect (*c*')	.0730	.0674	1.0834	.2899	-.0664	.2124
	Indirect effect ($a_i x b_i$)	-.0366	.0367			-.1083	.0416

Note: a_i represents the association between *GPER*-m and VMS; b_i represents the association between VMS and sleep quality; *c*' represents the direct effect of *GPER-m* on *Y*, after adjusting for all other variables included in the model; $a_i x b_i$ represents the indirect effect of *GPER-m* on sleep quality through VMS; *b* represents the unstandardized regression coefficient.
Abbreviations: se= standard error, BootLLCI= bootstrapping lower limit confidence interval; BootULCI= bootstrapping upper limit confidence interval; VMS= vasomotor symptoms, *GPER-m*= G protein-coupled estrogen receptor gene methylation; CV= control variables; MS= menopausal status; BMI= body mass index; E2= estradiol.

Likewise, when testing the effect of *GPER* methylation on the 3-factor model of the PSQI, the direct effect of *GPER* methylation on the global scores of perceived sleep quality, sleep efficiency and daytime dysfunction was not significant (b= 0.038, t(23)= 0.84, 95% CI [−.0544, 0.1295]), and nor was the indirect effect through VMS (b= −.023, 95% CI [−.0722, 0.0287]).

3.2.4 Discussion

In the present study, we examined the associations between *ESR1*/*GPER* methylation and sleep problems in healthy women aged 40–73 years. No direct associations between *ESR1*/*GPER* methylation and sleep problems were detected. However, we found that increased levels of *ESR1* methylation were predictive of increased severity of VMS, which in turn predicted increased sleep problems. These associations were significant even if potential cofounders such as age, MS, E2, and depressive symptoms were controlled (Cappuccio et al., 2008; Field et al., 2018; Gold et al., 2006; Hollander et al., 2001; Kravitz et al., 2008; Landis and Moe, 2004; Levine et al., 2016; Matsuda, 2014; Miner and Kryger, 2017; Roehrs and Roth, 2008; Woods and Mitchell, 2010).

The idea that epigenetic modifications of ER genes may be key drivers of the onset and emergence of perimenopausal symptoms, such as VMS, has been previously suggested (Brinton et al., 2015). One potential mechanism contributing to altered epigenetic patterns of ER genes may be the lifetime action of social and environmental determinants. For instance, early exposure to social stressors and environmental factors (eg: chemicals, nutrition) has been linked to altered *ESR1* methylation (Champagne et al., 2006; Doshi et al., 2011; Fiacco et al., 2019; Vrtačnik et al., 2014). Most interestingly, early life adversities, such as abuse and neglect, have been previously linked to increased *ESR1* methylation in rat (Champagne et al., 20006) and women (Fiacco et al., 2019), as well as to increased VMS among midlife women (Thurston et al., 2008).

The Study of Women Across the Nation (SWAN), assessing sleep quality in a multi-ethnic sample of 3’045 women in the US, showed that the prevalence of sleep disorders ranged from 16% to 42% in premenopausal women, from 39% to 47% in perimenopausal women, and from 35% to 60% in postmenopausal women, with Caucasian women reporting the highest rates (Kravitz et al., 2011). In our study, sleep problems with PSQI scores >5 were reported by 24% of premenopausal women, 31.3% of perimenopausal women and 35.5% of postmenopausal women. The characteristics of our entirely healthy sample may explain the slightly lower prevalence of poor sleep identified in the present study (Pengo et al., 2018). This may also contribute to explain the smaller direct effect of *ESR1* methylation on sleep problems, compared to the indirect effect mediated by VMS. The prevalence of VMS among the participating women was comparable with the results reported by the SWAN, which indicated that up to 80% of peri- and postmenopausal women experience VMS (Gold et al., 2006). In both the present study and previous studies, VMS were strongly associated with sleep problems (Baker et al., 2018). Together, these results may point to VMS as a primary cause of sleep problems also in healthy women.

The assessment of epigenetic modifications in peripheral tissues, such as blood, is increasingly seen as an important source of biomarkers in health and disease. In addition, as epigenetic signatures are mitotically heritable and yet reversible, their correction holds considerable therapeutic potential (Kronfol et al., 2017; Rasool et al., 2015). The clinical implications of *ESR1* methylation in VMS may merit further investigation. For instance, longitudinal studies which assess the status of *ESR1* methylation before the perimenopausal phase, and which monitor the development of VMS, may provide information regarding the potential of *ESR1* methylation as a predictive marker of VMS. In the present study, we used DNA extracted from

peripheral blood collected on dried blood spots (DBS). The DBS technology has been suggested as a promising tool in the context of research on biomarkers (Fischer et al., 2019). Its advantages include long-term storage of samples even at room temperature, and ease of transportation (Mei and Lee, 2014).

Although VMS may be primarily neurological symptoms involving brain regions such as hypothalamic areas, studying DNA methylation in the brain relies on post-mortem tissues, which has several implications in terms of impracticality (Freedman and Subramanian, 2005; Rhein et al., 2015). Therefore, peripheral tissues such as blood, buccal cells and saliva are currently used as a proxy of the brain in methylation studies (Fiori and Turecki, 2016; Zhang et al., 2013). Studies using peripheral tissues as a surrogate have demonstrated how specific methylation signatures are associated with disorders affecting the brain (Bakulski et al., 2016). In addition, although DNA methylation is tissue-specific, it has been demonstrated that at some CpGs, DNA methylation variance is more closely linked to individual specificity than to tissue specificity (Hannon et al., 2015). Online tools have been developed which enable a comparison of inter-individual methylation variance between peripheral tissues and the brain (Braun et al., 2019; Edgar et al., 2017; Hannon et al., 205). Using the online tool provided by Hannon et al (2015), we found that methylation of CpGs located in the assessed DNA region of *ESR1* was strongly correlated between blood and brain in the same individuals (Gardini et al., 2020). Therefore, methylation variations in blood may reflect methylation variations in the brain, for the assessed CpGs of *ESR1*.

Strengths and Limitations

To the best of our knowledge, this is the first study to examine and highlight associations between *ESR1* methylation and sleep problems through VMS in healthy middle-aged and older women. In this study, we assessed subjective sleep through the PSQI (Buysse et al., 1989; Pilz et al., 2018), while objective parameters of sleep were not measured. Indeed, questions have been raised regarding the validity of laboratory sleep assessments in women experiencing VMS (Ameratunga et al., 2012). For instance, evidence supporting an association between objective measures of sleep and VMS is limited and contradictory, despite the association between sleep problems and VMS is well known (Joffe et al., 2010). However, as subjective and objective sleep measurements may capture different aspects of sleep (Ameratunga et al., 2012; Landry et al., 2015), objective sleep assessments, such as polysomnography or actigraphy, may be needed to better elucidate the nature of the association between ER genes methylation and sleep.

Our sample included only healthy Caucasian women. On the one hand, as both the race and health status may affect subjective sleep difficulties and self-perceived menopausal symptoms (Baker et al., 2018; Kravitz et al., 2008; Kravitz and Joffe, 2015), the characteristics of our sample may help to limit biases linked to race and disease factors. On the other hand, our findings may not be generalizable to other racial/ethnic groups and to the general population including women with poor health. In addition, the sample of this study included three menopausal groups, and the distribution of participants among the three groups was not equal. In particular, the perimenopausal group included significantly fewer participants than the pre- and postmenopausal groups. These considerations may limit comparison with other studies. Furthermore, the sample size of *GPER* methylation, which was strongly reduced

compared to the sample size of *ESR1* methylation. Therefore, the assessment of *GPER* methylation in a larger sample may be useful in order to compare the relative contribution of *ESR1* and *GPER* methylation in the associations with sleep problems and VMS. Finally, the fact that we did not adjust for cell-type composition may also constitute a limitation of this study, as cell-type composition potentially affects levels of methylation in peripheral blood (Houseman et al., 2015). In conclusion, the main result of this study indicates that increased *ESR1* methylation may be associated with VMS, which in turn contribute to increased sleep problems in healthy middle-aged and older women. Therefore, this study supports the role of *ESR1* in middle-aged and older women's sleep problems through epigenetic mechanisms. Clinical implications, such as the potential of *ESR1* methylation as a biomarker of VMS, may be evaluated in future investigations.

3.2.5 Acknowledgments

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4. General Discussion

4.1 Summary of the findings

Using DBS and targeted- bisulfite NGS it was possible to measure DNA methylation at the regulatory regions of ER genes. However, the number of samples in which *GPER* methylation was correctly measured was much lower compared to *ESR1*. Levels of methylation were low at the CpG island of promoters, while intermediate levels of methylation were found at the *ESR1* shore of promoter C. The aim of the first study was to verify whether levels of methylation of key regulatory regions of the *ESR1* were associated with menopausal status, age, and E2 levels. We found that lower E2 levels, and not age, were associated with lower methylation of the *ESR1* shore, particularly at three specific CpGs. These CpGs are potentially involved in the negative feedback mechanism underlying the regulation of ERα by E2 (Ianov et al., 2017). One of these CpGs (CpG9) was hypomethylated in postmenopausal women compared to premenopausal women. Increased methylation of one CpG of the proximal promoter B was associated with age, but not with E2 or menopausal status. The aim of the second study was to verify whether levels of *ESR1* and *GPER* methylation were associated with phenotypes related to dysfunctional estrogen signaling in the SCN, such as sleep problems and vasomotor symptoms. We found that increased levels of the *ESR1* shore methylation (and not of *ESR1* promoter A and B, data not shown) were predictive of increased severity of VMS, which in turn predicted increased sleep problems. The direct association between *ESR1* methylation on sleep problems was less pronounced, while there was no evidence regarding the role of *GPER* methylation in sleep problems and VMS.

Although the targeted bisulfite NGS for the analysis of *GPER* methylation was correctly optimized, there were several missing data in the final results. Missing data on *GPER* methylation may be due to the loss in DNA stability following repeated freeze-thawing cycles of DBS, extracted DNA, and converted DNA that occurred before the analysis of *GPER* (Li and Tollefsbol, 2011; Sjoholm et al., 2007). Consistent with the literature on DNA methylation, the CpG island of promoters had low levels of DNA methylation (Moore and Fan, 2013). Instead, higher and intermediate levels of methylation were found at the *ESR1* shore of promoter C. Based on its structure and function, this DNA region was previously classified as an enhancer element (ID GH06J151804; Fishilevich et al., 2017; Tsuboi et al., 2017). Active enhancers are characterized by intermediate levels of methylation, which indicate that the shore of promoter C analysed in blood in the context of this thesis was an active enhancer (Magnusson et al.,2015). Previous works suggested that clock CpGs are localized to promoters and enhancers. Methylation changes at clock CpGs can result in beneficial or detrimental effects on the health status of an aging organism (Jones et al., 2015; Ashapkin et al., 2017; Avrahami et al., 2015; Ciccarone et al., 2018). Hypermethylation at the proximal *ESR1* promoter A and B has been associated with age and with increased risk of disease (Issa et al., 2002; Lv et al., 2011). The results of the first study indicated that methylation at only one CpG in promoter B was associated with age, while the remaining CpGs in promoter B and promoter A were not. As women in this study were healthy, it is possible that the weak association between methylation of proximal promoters and age found in this study is a marker of healthy aging. If, on one hand, hypermethylation of promoters during aging may increase the risk of disease, on the other hand, hypomethylation at enhancers may result in beneficial effects on the

health status of aging organisms and may be determined by levels of sex steroids (Ciccarone et al., 2018; Ianov et al., 2017; Jylhävä et al., 2017; Tsuboi et al., 2017). Therefore, our results showing lower levels of E2 associated with hypomethylation of the *ESR1* enhancer in healthy middle-aged and older women may constitute another marker of healthy aging. However, as aging cannot be programmed, this association should not be regarded as an "aging program". Methylation changes at the *ESR1* enhancer beyond the fertile life may correspond to a developmental and reproductive program that is not switched off after menopause (Blagosklonny, 2013; Fabian and Flatt, 2011). In support of this argument, the positive correlation between *ESR1* enhancer methylation and E2 was also identified in the premenopausal women. During the fertile life, this mechanism could contribute to the negative feedback that regulates levels of ERα in response to E2 fluctuations, in order to maintain a balanced ERα signaling (Liu and Shi, 2015; Ianov et al., 2017). Consistently, the continuation of this program beyond the fertile life would imply lower *ESR1* enhancer methylation, in order to deal with lower E2 levels. This would contribute to maintain the ERα signaling during postmenopause and thereby reduce the risk of age-related health disorders. This hypothesis is supported by the second study of this thesis, which indicated that *ESR1* enhancer hypomethylation was associated with decreased VMS and sleep problems in peri- and postmenopausal women. These symptoms have been associated with dysfunctional ERα signaling in the women SCN, which is a hypothalamic region maintaining vital physiological functions in the entire human body (Albertson and Skinner, 2010; Brinton et al., 2015; Gouw et al., 2017; Hatcher et al., 2018; Kim and Choe 2018). Therefore, the hypomethylation of the *ESR1* enhancer may be a key contributor to the preservation of health in aging women. It should be noted that methylation of CpGs in the *ER* gene regions assessed in these studies was the best correlated between blood and brain at CpGs located in the *ESR1* enhancer. Therefore, for the CpGs located in this region, methylation variations in blood may reflect methylation variations in the brain. This does not exclude that methylation at other CpGs is associated with VMS and sleep, as DNA methylation is generally tissue-specific.

In the women 40+ healthy aging study, 16 perimenopausal women were recruited and were included in the present thesis. In this group of women, showing the highest VMS and sleep problems, there was no evidence for a positive association between *ESR1* enhancer methylation and E2 levels, which could contribute to balancing the estrogen signaling. According to Brinton et al., (2015), dysregulation of the estrogen signaling, including epigenetic alterations of the ERs, leads to the neurological symptoms (e.g. VMS, insomnia, mood changes, memory impairments) characterizing the perimenopause (Brinton et al., 2015). Although the perimenopause was not the primary focus of this thesis, the finding on the altered association between E2 and *ESR1* enhancer methylation in perimenopausal compared to pre-and postmenopausal women may be related to the exacerbation of menopausal symptoms.

4.2 Conclusion and direction for future research

Life expectancy is currently increasing more rapidly than healthy life expectancy, especially for women. This highlights the need for research to understand the mechanisms underlying women healthy aging to prevent age-related diseases. Declining E2 levels associated with menopause correlated with an increased incidence of age-related health disorders, suggesting that E2 is protective for women health. Most of the beneficial effects of E2 are

mediated by the ERs. Among the three mains characterized ERs, the ERα, ERβ, and GPER, ERα has emerged as the most important in maintaining women health during aging. Increased levels of ERα have been shown to be protective against dysfunctions of key hypothalamic regions that accelerate the aging process of the entire body, and against the progression of various age-related diseases, such as Alzheimer and Parkinson's disease, osteoporosis, and cardiovascular diseases. DNA methylation of the ER genes is a key regulator of the levels of ERs. Evidence indicates that the regulation of enhancer and promoter elements of the ERα gene (*ESR1*) may be relevant for modulating health in aging women. In particular, animal and human research indicates potential regulation of DNA methylation at enhancers by E2 that could promote healthy aging, and increased risk of disease during aging associated with hypermethylation of the regulatory regions of *ESR1*.

This thesis demonstrated that in the blood of healthy middle-aged and older women, decreased methylation of *ESR1* enhancer was associated with conditions of lower E2, and that increased methylation of this sequence was associated with VMS and sleep problems. In addition, hypermethylation at proximal promoters, which is associated with age and increased risk of disease in the general women population, was less pronounced in healthy women. Finally, this study demonstrated that the DBS technology is suitable for exploring associations between methylation of ER genes and women health during aging.

Future research should aim to confirm and advance these findings. First, the assessment of *GPER* methylation should be included in future studies on women healthy aging, as several rapid effects of ERα may be GPER-mediated. Second, the assessment of intra-individual correlation of DNA methylation at the key ER gene regions between blood and other tissues may be useful to evaluate the utility of peripheral blood as a surrogate. Third, ER genes methylation from DBS should be assessed in the context of longitudinal studies targeting the general population, in order to establish clinical biomarkers providing information on the current health status and the risk of developing unhealthy conditions during aging. Fourth, the association between the *ESR1* enhancer methylation and E2 should be explored further. The confirmation of a negative feedback mechanism through *ESR1* enhancer methylation could contribute to better understanding the regulation of ERα by E2 levels. Finally, healthy aging studies should integrate the determination of genetic polymorphisms *XbaI*, *PvuII*, and TA repeat, to explore their association with DNA methylation changes of the *ESR1* enhancer during aging. Indeed, these polymorphisms could contribute to increased *ESR1* methylation, and therefore, to increase the risk to develop disabilities during aging.

In summary, this thesis highlighted potential *ESR1* methylation mechanisms that would underly women healthy aging.

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Appendix

DNA collection on Dried blood spots (DBS)

S&S 903 Whatman® paper cards (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom)

Material:

- Alcoholic pads: Soft-Zellin 60x30mm, 100 pcs, REF: 999 979, Hartmann
- Niedles: Accu-Chek® Safe-T-Pro Plus (Ref: 158964) Roche
- Cellulose wadding pads: Zellstoffwatte-Tupfer, 110xREF 285450, Mediset, Hartmann

- Whatman 903 protein saver card (#28416622)
- Patches: Mefix, 5 cm x 10m/2 in x11 yd, REF 310500, Mefix
- Enveloppes: Env. Glassine, 100 pcs, REF 10548236, Whatman
- Dessicant: Dessiccant 1000, REF WB100003, Whatman

Collection procedure:
COLLECTION

- Wash hands thoroughly with soap and warm water, then dry hands.
- Use the provided alcohol pad to clean the tip of the finger (ring finger or middle finger).
- Break the seal on the lancet.
- Position the lancet against the inside tip of the finger. Press down firmly until an audible click is heard.
- Gently press the pricked finger from below the puncture site to allow for a large drop of blood to form. Important: Wipe away the first drop of blood with provided gauze.
- Apply a hanging drop of blood. Apply the blood drops within each circle on the Blood Spot Collection Card.

Drying and packing procedure

- Air dry completely. Prop the card up and let air dry for at least 3 hours.
- Keep specimen away from moisture and sunlight.
- Once fully dried, tuck the cover into flap as indicated. Insert the Blood Spot Collection Card, Desiccant, and the Humidity Indicator into the silver bag and seal tightly. Each Silver bag holds a maximum of 5 cards. Important: Do not fold the Blood Spot Collection Card.

DNA extraction from Dried blood spots (DBS)
Kit: QIAamp DNA Investigator Kit (QIAGEN, Hilden, Germany)

DNA extraction from Dried blood spots (DBS).
Kit: QIAamp DNA Investigator Kit (QIAGEN, Hilden, Germany)

Material:

- Punch: Uni-Core Punch 3.0mm, 4 pcs, REF WB100039, Whatman
- Cutting mat: Harris Micro punch replacement cutting mat, REF WB100020, Whatman
- QIAamp DNA Investigator Kit (50), REF 56504, Qiagen
- Ethanol in rack
- Javel (diluted 70%) in rack
- Zellstoffwatte
- Eppendorf 1.5

Protocol: QIAamp® DNA Investigator Handbook - Qiagen, p. 17
Perform all centrifugation steps at room temperature (15–25°C)

To do before starting the analysis (ex.: when kit arrives): AW1, AW2, carrier RNA preparation (instructions on page 11).

Preparing Buffer AW1
Add 25 ml ethanol (96–100%) to the bottle containing 19 ml Buffer AW1 concentrate. Tick the check box on the bottle label to indicate that ethanol has been added. Reconstituted Buffer AW1 can be stored at room temperature (15–25°C) for up to 1 year.
Note: Before starting the procedure, mix the reconstituted Buffer AW1 by shaking.

Preparing Buffer AW2
Add 30 ml ethanol (96–100%) to the bottle containing 13 ml Buffer AW2 concentrate. Reconstituted Buffer AW2 can be stored at room temperature (15–25°C) for up to 1 year.
Note: Before starting the procedure, mix the reconstituted Buffer AW2 by shaking.

Preparation of carrier RNA: For purification of DNA from very small amounts of sample, such as low volumes of
blood (<10 µl) or forensic samples, we recommend adding carrier RNA to Buffer AL. For samples containing larger amounts of DNA, addition of carrier RNA is optional.
Add 310 µl Buffer ATE to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl. Dissolve the carrier RNA thoroughly, divide it into conveniently sized aliquots, and store at –20°C. Do not freeze–thaw the aliquots of carrier RNA more than 3 times.
If Buffer AL or Buffer ATL contains precipitates, dissolve by heating to 70°C with gentle agitation
Set incubator (heating block) to 56°C

1. Cut 3 punches (3mm) from a dried spot with a Uni-Core punch. Place the 3 punches into a 1.5 ml microcentrifuge tube.
2. Add 280 µl Buffer ATL.
3. Add 20 µl proteinase K and mix thoroughly by vortexing.

4. Place the 1.5 ml tube in the heating block and incubate at 56°C for 1 h. Vortex the tube for 10 s every 10 min.
5. Set the heating block to 70°C.
6. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.
7. Add 1 ul of carrier RNA to 300 ul of buffer AL/per sample. Example for 10 samples (3000 ul buffer AL with 10 ul carrier RNA).
8. Add 300 µl Buffer AL-carrier RNA to the samples, close the lid, and mix by pulse-vortexing for 10 s. To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogeneous solution.
9. Place the 1.5 ml tube in the heating block and incubate at 70°C for 10 min. Vortex the tube for 10 s every 3 min. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.
10. Add 150 µl ethanol (96–100%), close the lid, and mix thoroughly by pulse vortexing for 15 s. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid.
11. Carefully transfer the entire lysate from step 10 to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim, close the lid, and centrifuge at 8000 rpm for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
12. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 8000 rpm for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
13. Carefully open the QIAamp MinElute column and add 700 µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 8000 rpm for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through. Avoid contact of the column with the flow-through.
14. Carefully open the QIAamp MinElute column, and add 700 µl of ethanol (96–100%) without wetting the rim. Close the cap and centrifuge at 8000 rpm for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
15. Centrifuge at full speed 13,000 rpm for 4 min to dry the membrane completely.

16. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube, and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column, and incubate at room temperature (15–25°C) for 10 min.
17. Apply 30 µl of distilled water to the center of the membrane.Incubate at room temperature for 5 min. Centrifuge at full speed for 2 min.
18. Measure DNA with nanodrop. DNA should be around 20 and 40 ng/ul which will drop down at 5 ng/ul after bisulfite conversion.

Bisulfite treatment of genomic DNA

EZ-96 DNA Methylation-Gold™ Kit Catalog No. D5008 (Deep-Well Format)

Material

- CT Conversion Reagent* 2 bottles Room Temp.
- M-Dilution Buffer 7 ml Room Temp.
- M-Dissolving Buffer 1.2 ml Room Temp.
- M-Binding Buffer 125 ml Room Temp.
- M-Wash Buffer** 2 x 36 ml Room Temp.
- M-Desulphonation Buffer 40 ml Room Temp.
- M-Elution Buffer 8 ml Room Temp.
- Zymo-Spin™ I-96 Binding Plates 2 plates Room Temp.
- Conversion Plates w/ Pierceable Cover Film 2 plates/films Room Temp.
- Collection Plates 2 plates Room Temp.
- Elution Plates 2 plates Room Temp

Procedure

1. Add 130 µl of the CT Conversion Reagent to 20 µl* of each DNA sample in a Conversion Plate. If the volume of the DNA sample is less than 20 µl, make up the difference with water. Mix the samples by pipetting up and down.
2. Seal the plate with the provided film. Transfer the Conversion Plate to a thermal cycler and perform the following steps:

    1. 98°C for 10 minutes
    2. 64°C for 2.5 hours
    3. 4°C storage for up to 20 hours

3. Add 600 µl of M-Binding Buffer to the wells of a Zymo-Spin™ I-96 Binding Plate mounted on a Collection Plate.
4. Transfer the samples from the Conversion Plate (Step 2) to the wells of the ZymoSpin™ I-96 Binding Plate. Mix by pipetting up and down.
5. Centrifuge at ≥ 3,000 x g (5,000 x g max.) for 5 minutes. Discard the flow-through.
6. Add 400 µl of M-Wash Buffer to each well of the plate. Centrifuge at ≥ 3,000 x g for 5 minutes.
7. Add 200 µl of M-Desulphonation Buffer to each well and allow the plate to stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at ≥ 3,000 x g for 5 minutes. Discard the flow-through.
8. Add 400 µl of M-Wash Buffer to each well of the plate. Centrifuge at ≥ 3,000 x g for 5 minutes. Discard the flow-through. Add another 400 µl of M-Wash Buffer and centrifuge for 10 minutes.

9. Place the Zymo-Spin™ I-96 Binding Plate onto an Elution Plate. Add 15 µl of water directly to each well. After 5 minutes, centrifuge at ≥ 3,000 x g for 3 minutes to elute the DNA. The DNA is ready for immediate analysis or can be stored at or below -80°C for later use.

Library preparation

1. DNA amplification

Universal primers	Sequence	Length (bp)
Cs1	ACA CTG ACG ACA TGG TTC TAC A NNN	25
Cs2	TAC GGT AGC AGA GAC TTG GTC T NNN	25

Note: specific primers for the desired DNA target are combined to the universal primers CS1/CS2

Master Mix

Product	Volume (ul)
Kapa U+	5
Primer F (10uM)	0.5
Primer R (10uM)	0.5
H2O	3
Bs DNA	1

Cycling conditions

	Initial denaturation	95 °C	3 min
40 x	Denaturation	98°C	20 sec
	Annealing	60°C	15 sec
	Extension	72°C	15 sec
	Final extension	72°C	30 sec

2. Purification of the PCR products

Material

- E-Gel® SizeSelect™ Agarose Gels, 2%_Thermo Fisher_ Cat No: G661002 267 CHF_1 pck (10 e-gels_ 8 sample/gel).
- 50 bp DNA Ladder_Thermo Fisher_Cat No: 10416014 _ 50 µg
- Invitrogen™ E-Gel™ Safe Imager

Procedure

1. Add 15ul H2O to the samples
2. Add 25ul H2O in all empty spaces
3. Load the samples, if ladder 6 ul H2O + 4 ul ladder
4. Run for 15 min 30 sec, chosing the program 9:egel size selection
5. Refill with water the empty spaces (10-15 ul)
6. Run till samples collection

Total volume of purified DNA is 20-25 ul. Store at -20°C

3. Barcoding of the purified PCR products

Access Array Barcode Library for Illumina Sequencers - 384 (Single direction) 100-4876 (Fluidigm, San Francisco, CA, USA)

Product	**Volume (ul)**
Kapa U+	5
Barcode (2uM)	1
H2O	2
Purified DNA	2

Cycling conditions

	Initial denaturation	95 °C	3 min
10 x	Denaturation	98°C	20 sec
	Annealing	60°C	15 sec
	Extension	72°C	15 sec
	Final extension	72°C	40 sec

4. Pooling (if multiple amplicons)

1. Calculate the concentration for each amplicon for a molarity of 2nM, knowing the length of each amplicon. Pool the amplicons at the same molarity.
Molarity (2nM)= concetration(ng/ul) * 10^6 / 660*Avarage library fragments lengths
2. Final purification: Take 25 ul of the library and run it on E-gel_size selection as usual. Collect the purified product and dilute it 5 x.

Sequencing

1. Quantify the library with Tape station- Agilent 2200 TapeStation-High Sensitivity D1000 ScreenTape Assay

- Bring products at room temperature for 30 minutes.
- Vortex products and library
- In the first tube: DHS 1000 Ladder (2ul) + DHS1000 sample buffer (2ul)
- In the other tubes, library (2ul) + DHS1000 sample buffer (2ul)
- Close caps of tubes and vortex for 1 min
- Briefly centrifuge the tubes
- Take the caps off and place the samples, tips, agilent columns in the tape station
- Save the file and start the program

2. Dilute the PhiX at 2nM: 5ul PhiX (10nM) + 20 ul RSB

3. Mix: 8 ul of library at 2 nM + 2 ul of PhiX at 2 nM

4. Mix: 5 ul of Library + PhiX mixed with 5 ul of 0.2N NaOH. Incubate at room temperature for 5 minutes. To prepare 0.2N NaOH from 1N NaOH stock: 800 ul grade water + 200 ul NaoH1N

5. Add 990 ul of cold HT buffer

6. Take 600 ul and add 400ul HT

7. Load 600 ul in tube 17 of Miseq cartridge Final= 10pM